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SPECIES-SPECIFIC COMPOSITION OF FREE AMINO ACIDS ON THE LEAF SURFACE OF FOUR *Senecio* SPECIES

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Abstract—The specificity of the chemical composition of the leaf surface of four *Senecio* species was studied in order to understand the host selection of *Tyria jacobaeae*, a monophagous moth that has *Senecio jacobaea* as its principal host plant. Pyrrolizidine alkaloids characteristic for *Senecio* species were not detected in water and sulfuric acid extracts of the leaf surface of *Senecio jacobaea*. Water extracts of the leaf surfaces of *S. jacobaea*, *S. vulgaris*, *S. viscosus*, and *S. sylvaticus* contained low concentrations of free amino acids. The proportions of these amino acids were very different from those inside the leaf tissues. In a discriminant analysis, the four species could be completely separated on the basis of the relative proportions of amino acids on the leaf surface.

Key Words—Amino acids, leaf surface, chemotaxonomy, phylloplane, pyrrolizidine alkaloids, *Senecio jacobaea*, *Senecio sylvaticus*, *Senecio viscosus*, *Senecio vulgaris*, *Tyria jacobaeae*.

INTRODUCTION

Host-plant recognition by phytophagous insects cannot usually be explained by the presence or absence of a single stimulus, but rather by a combination of many stimuli (Fox and Lalonde, 1993; Renwick and Chew, 1994). The chemical

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subset of these stimuli or "chemical search image" plays an important role in host plant recognition (Städler, 1986). For ovipositing insects that do not contact the inner tissues of the plant, but test only the leaf surface, recognition and selection could be determined by small quantities of many types of chemical substances that come from the inner tissues and that are present on the plant surface; for example, free amino acids, organic acids, sugars, secondary metabolites, vitamins, minerals, and growth regulators (Tukey, 1971; Fokkema, 1981; Städler, 1986; Derridj et al., 1989, 1992, 1996; Fiala et al., 1990; Städler and Roessingh, 1990; Jackson et al., 1991; Roessingh et al., 1992). These substances, especially secondary metabolites, could give species-specific information to a phytophagous insect. Many secondary metabolites, however, are large molecules that do not diffuse through membranes and cell walls easily, and thus are not likely to be present on the leaf surface. Primary metabolites, on the contrary, are probably present on the leaf surface of all plants (Tukey, 1971).

Recent developments in chemotaxonomy show that plants can be discriminated on the basis of their internal proportions of primary substances. Laatsch et al. (1993) could separate families of fungi on the basis of their proportions of protein amino acids, and Yeoh et al. (1984) described species-specific amino acid proportions in leguminous plants. It has already been shown that proportions of free amino acids in the phylloplane of maize and sunflower are very stable and specific (Derridj et al., 1989). It is tempting to suggest, therefore, that primary metabolites play a role in interspecific host-plant recognition of phytophagous insects. In this paper we examine this subject by studying the uniqueness of proportions of free amino acids on the leaf surface of *Senecio jacobaea* L., the primary host plant of the monophagous moth *Tyria jacobaeae* L. (Lepidoptera, Arctiidae), by comparing them to the proportions on the leaf surface of *S. vulgaris*, a less preferred host, and *S. viscosus* and *S. sylvaticus*, two nonhosts.

The *Senecio–Tyria* system was chosen for three reasons. Firstly, the microbehavior of females clearly shows that chemical factors playing a role in oviposition must be present on the leaf surface. The females continuously touch the leaf surface with their antennae and most of them (but not all) drum on the leaf with their back tarsae. These tarsae do not contain spines that could penetrate through the cuticle, but contain a large number of contact chemoreceptors. Secondly, we think that the characteristic pyrrolizidine alkaloids (PAs) of *Senecio* species do not play a direct role in oviposition. Neither oviposition nor larval performance seems to be influenced by the concentrations of PAs in the leaf tissues of *S. jacobaea* (van der Meijden et al., 1989; Soldaat and Vrieling, 1992; Vrieling et al., 1991). However, the few food plants known for *Tyria* all contain PAs, indicating that PAs or PA-related factors are present on the leaf surface, where the only contact between female and plant takes place. Such related factors could be the amino acids arginine or ornithine, which are pre-

cursors of PAs. The third reason to study the *Senecio–Tyria* system is that oviposition preference is positively related to the concentration of organic nitrogen in the plant tissues (van der Meijden et al., 1989). Amino acids on the leaf surface are the most likely cue to assess organic nitrogen concentrations inside the plant.

To examine the hypothesis that primary metabolites may be concerned in the host specificity of *Tyria jacobaeae*, we first had to study the presence of pyrrolizidine alkaloids on the leaf surface and to investigate whether it is possible to discriminate between different *Senecio* species on the basis of the amino acids on their leaf surfaces.

METHODS AND MATERIALS

Seeds of *S. jacobaea* came from plants in the sand dunes of Meijendel (near the Hague, the Netherlands), seeds of *S. vulgaris* were collected in an experimental field of the INRA at Versailles and seeds of *S. sylvaticus* and *S. viscosus* were supplied by the National School of Horticultural Science (ENSH) at Versailles. The seedlings of each species were placed in 11 pots, filled with a 1 : 1 mixture of perlite and compost and cultivated in a greenhouse. The pots were saturated with nutrient solution for 5 min three times a day. The conditions in the greenhouse were: ambient photoperiod and light, relative humidity 70% ± 10%, maximum daytime temperature 25°C and minimum nightime temperature 15°C. Leaf samples for analysis were cut in the middle of the day in order to minimize variation between replicates due to circadian rhythms in the plants. Leaf area in all experiments was determined using a photoplanimeter.

Amino Acids on Leaf Surface

Experiment 1. In this experiment the four plant species were grown and analyzed successively through the year. *S. jacobaea* was analyzed in November 1992. Four replicates were performed; each replicate consisted of three plants of which two leaves were washed. *S. vulgaris* was analyzed in May 1993; three replicates were performed, each consisting of three plants of which three leaves were washed. *S. viscosus* was analyzed in March 1993; three replicates were performed, each replicate consisting of three plants of which two leaves were washed. *S. sylvaticus* was analyzed in May 1993; again, three replicates were performed, each consisting of one plant of which two leaves were washed, because of the limited number of suitable plants. The number of genotypes per replicate was therefore 3, 3, 3, and 1 for the four species, respectively. As *S. jacobaea* is a biennial plant species, the cultivation of flowering plants takes several months. Therefore, we used middle-aged leaves of rosette plants, while for the other three annual species, leaves halfway up the flowering stem were

used. All plants were 2–3 months old when sampled. All the phenological stages mentioned can be found in the field when oviposition of *T. jacobaeae* takes place.

Experiment 2. In this experiment the four species were analyzed simultaneously. The growing conditions and the growth stages of the plants at the time of sampling were the same as the first experiment. The experiment was performed in November 1993 on three successive days. On each day we sampled and sprayed four leaves of one plant for three species and eight leaves for *S. vulgaris*, which has smaller leaves than the other three species. In this experiment each replicate consisted of one genotype.

Leaf Washing

Amino Acids. Leaves were cut from the plants and weighed. The petioles were packed in aluminum foil to prevent contamination of the leaf surface by the leaking of substances from the inner tissues. Then the leaves were sprayed with 20–25 ml of ultrapure water during 25–30 sec, following the technique described by Fiala et al. (1990). The upper surface and the lower surface of the leaves were sprayed separately. After filtration through a 0.45-μm Millipore filter and evaporation, the leaf washings were stored at $-80°C$. In the second experiment the washings of both sides of the leaves were collected together in order to enlarge the quantities of substances in each sample.

Alkaloids on Leaf Surface of Senecio jacobaea. Leaves of 3-month-old rosette plants of *S. jacobaea* were dipped vertically for 30 sec in a narrow plastic container filled with 600 ml solvent. The youngest and oldest leaves of a plant were not used. During dipping the leaves were held by the cut petioles, which prevented contamination of the solvent with substances from the inner leaf tissues. The leaves were lightly agitated up and down. The following solvents were used: (1) water to which 10% ethanol was added to increase contact between the water and the leaf surface, (2) 0.005 M H_2SO_4; (3) 0.05 M H_2SO_4; and (4) 0.5 M H_2SO_4. Sulfuric acid was added because pyrrolizidine alkaloids solve easily in acid solutions. A total of 60 leaves was dipped in each solution (six plants, 10 leaves per plant). This experiment was repeated on three successive days, which brought the total number of analyzed leaves to: 3 (replicates) $\times$ 4 (solutions) $\times$ 6 (plants) $\times$ 10 (leaves) = 720 leaves. The solutions were filtered through a 0.45-μm Millipore filter, and stored at $-30°C$. Methanol was added to prevent the solutions from freezing.

Amino Acids Inside Leaf Tissues

For *S. jacobaea* only, we determined the concentrations of free amino acids inside the leaves in the first experiment. After spraying, the leaves were frozen in liquid nitrogen, freeze-dried, ground, and stored dry until chemical analysis.

Chemical Analyses

Amino Acids. Free amino acids were determined using a Biotronik LC 5001 analyzer (physiological program run with lithium buffers, detection at 570 and 440 nm after postcolumn derivatization with Ninhydrin) and the PE Nelson 2100 software.

Pyrrolizidine Alkaloids. The solutions were macerated for 2 hr with excess zinc powder to reduce N-oxides and filtered over a Büchner funnel supplied with a paper filter and made alkaline (pH > 10) with 0.5 ml 25% ammonium hydroxide. Then the solutions were passed through an Extrelut column (Merck) and after 20 min the PAs were recovered with 2× 15 ml dichloromethane (van Dam et al., 1993). After evaporation of the dichloromethane, the residue was redissolved in 5.0 ml methanol. The presence of PAs was determined spectrophotometrically in duplicate by a color reaction (Mattocks, 1967). Monocrotaline (Sigma, 99% purity) was used as a reference.

RESULTS

Amino Acids on Leaf Surface of Four Senecio *Species*

All protein amino acids (less cysteine) and also ornithine and GABA were present on the leaf surface of all four plant species (Table 1) in very low, but for insects detectable concentrations (Table 2). The concentrations of cysteine were too low to be accurately detected. Unknown peaks of Ninhydrin-positive compounds were found in the chromatograms of all species, but none of these compounds was specific for a species. The total concentrations of amino acids varied significantly between the four species and between the two experiments (Table 2). The significant interaction between species and experiment is caused by *S. sylvaticus*, which had relatively low concentrations in the first and high concentrations in the second experiment as compared to the other three species. For the rest, the same order in the total concentration of amino acids was found in both experiments, with *S. vulgaris* having by far the highest concentrations (Table 2). No significant differences were found in a pairwise *t* test between the concentrations on the upper and lower sides of the leaves of *S. jacobaea* in the first experiment (upper side: 2254 ± 570 nmol/m^2 (SE); lower side: 2183 ± 410 nmol/m^2; $N = 4$ pairs).

The proportions of free amino acids found on the leaf surface were roughly similar both between species and between experiments within a species (Table 1), except for some amino acids (especially glutamine, proline, ornithine, and arginine). For the proportions of each amino acid, we performed a two-way ANOVA, with experiment and species as factors. The significance level of 5% was corrected for the number of analyses performed (21), using the sequential

TABLE 1. PROPORTIONS OF FREE AMINO ACIDS IN LEAF TISSUES OF *S. jacobaea* AND ON LEAF SURFACES OF *S. jacobaea*, *S. vulgaris*, *S. viscosus*, AND *S. sylvaticus*[a]

			Leaf surface															
	S. jacobaea tissue		*S. jacobaea*				*S. vulgaris*				*S. viscosus*				*S. sylvaticus*			
	Exp. 1 ($N = 4$[b])		Exp. 1 ($N = 4$)		Exp. 2 ($N = 3$)		Exp. 1 ($N = 3$)		Exp. 2 ($N = 3$)		Exp. 1 ($N = 3$)		Exp 2 ($N = 3$)		Exp 1 ($N = 3$)		Exp. 2 ($N = 3$)	
Amino acid	%	Se	%	Se	%	Se	%	se	%	se	%	Se	%	Se	%	Se	%	Se
asp	17.45	0.60	7.12	0.46	5.50	0.28	8.01	0.75	6.47	0.41	7.20	0.37	5.70	0.10	7.98	1.29	6.52	0.49
thr	2.79	0.15	5.00	0.43	4.15	0.07	4.02	0.28	4.65	0.17	5.26	0.23	5.01	0.02	4.20	0.32	5.03	0.53
ser	17.39	1.42	21.03	2.86	19.08	1.74	19.08	1.50	25.07	1.12	26.50	0.66	25.84	0.13	17.16	0.83	24.05	4.46
asn	1.17	0.11	1.86	0.22	2.51	0.19	1.57	0.21	1.47	0.30	1.01	0.10	0.98	0.02	1.47	0.45	0.98	0.29
glu	21.51	1.44	4.66	0.34	5.04	1.06	7.24	1.43	2.29	0.47	3.11	0.14	1.63	0.04	6.18	1.89	2.97	0.40
gln	16.78	1.70	3.15	0.43	5.46	0.65	13.29	3.62	1.49	0.55	1.11	0.33	1.02	0.28	7.02	1.72	1.84	0.54
pro	2.25	0.45	3.28	0.34	4.02	0.23	3.57	0.51	2.71	0.24	5.70	0.45	2.45	0.06	10.59	0.58	2.25	0.38
gly	0.44	0.08	14.68	0.82	11.75	1.05	11.37	1.30	15.80	0.73	16.19	0.21	17.98	0.29	8.71	1.03	15.53	0.51
ala	13.54	0.64	10.83	0.22	9.43	0.52	10.87	0.38	10.30	0.13	11.11	0.13	11.63	0.13	12.78	1.74	11.25	0.17
val	0.83	0.06	3.28	0.49	2.99	0.10	2.89	0.27	3.37	0.16	2.74	0.29	3.10	0.07	3.17	0.34	3.12	0.78
met	0.10	0.02	0.19	0.06	0.25	0.02	0.16	0.07	0.63	0.21	0.09	0.05	0.34	0.02	0.49	0.40	0.36	0.11
ile	0.50	0.02	2.27	0.44	1.92	0.08	2.01	0.19	2.11	0.10	1.43	0.03	2.18	0.04	1.86	0.47	2.13	0.68
leu	0.53	0.09	2.77	0.29	2.73	0.28	2.25	0.35	2.69	0.08	1.81	0.08	2.67	0.08	1.98	0.37	2.82	0.26
tyr	0.29	0.04	1.62	0.31	1.69	0.04	1.19	0.10	1.87	0.15	1.92	0.22	1.94	0.10	0.97	0.13	1.91	0.51
phe	0.60	0.04	1.89	0.18	2.05	0.28	1.50	0.18	1.83	0.15	1.09	0.09	1.70	0.03	1.94	0.13	1.95	0.10
gaba	2.65	0.53	1.20	0.30	2.26	0.29	1.58	0.25	0.42	0.16	0.40	0.03	0.25	0.02	2.68	0.46	1.11	0.32
orn	0.30	0.02	7.12	1.09	6.53	0.70	5.06	0.93	9.20	0.25	5.80	0.91	9.49	0.40	3.80	0.55	8.67	1.08
lys	0.39	0.06	2.53	0.42	2.87	0.41	1.61	0.29	2.58	0.13	1.83	0.21	2.33	0.08	1.97	0.27	2.38	0.54
his	0.16	0.02	3.42	0.33	4.09	0.78	1.65	0.13	2.99	0.15	2.88	0.11	2.57	0.08	2.35	0.39	3.05	0.53
trp	0.00	0.00	0.00	0.00	0.37	0.17	0.33	0.15	1.10	0.18	0.50	0.20	0.71	0.03	0.00	0.00	0.66	0.16
arg	0.32	0.05	2.10	0.58	5.30	2.38	0.75	0.17	0.97	0.29	2.34	0.14	0.50	0.03	2.70	0.76	1.40	0.39

[a]asp = aspartic acid, thr = threonine, ser = serine, asn = aspartine, glu = glutamic acid, gln = glutamine, pro = proline, gly = glycine, ala = alanine, val = valine, met = methionine, ile = isoleucine, leu = leucine, tyr = tyrosine, phe = phenylalanine, GABA = gamma-amino butyric acid, orn = ornithine, lys = lysine, his = histidine, trp = tryptophan, arg = arginine. The leaf surface data in experiment 1 only concern the lower leaf side. In experiment 2 both leaf sides were mixed (see text). SE = standard errors (%).

[b]N is number of replicates.

TABLE 2. TOTAL CONCENTRATION OF FREE AMINO ACIDS ON LEAF SURFACE OF *S. jacobaea*, *S. vulgaris*, *S. viscosus* AND *S. sylvaticus*[a]

	S. jacobaea	*S. vulgaris*	*S. viscosus*	*S. sylvaticus*
Exp. 1	2.27	14.07	10.72	2.46
	2.48	17.91	6.82	1.20
	3.62	7.82	3.48	1.85
	1.68			
Exp. 2	4.03	34.25	5.99	9.22
	6.81	49.97	9.28	6.19
	7.39	18.38	8.27	5.29

ANOVA	*df*	MS	*F*	*P*
Species	3	521.9	14.78	0.000
Experiment	1	345.9	9.79	0.006
Species × exp.	3	124.2	3.52	0.038
Residual	17	35.3		
Total	24	121.9		

[a]Results are given in μmol/m^2 for all replicates, and results of a two-way analysis of variance on the data.

Bonferroni test (Rice, 1989). The proportions of proline, ornithine, and tryptophan were significantly different between the experiments. Only the proportions of proline, glycine, and GABA differed significantly between the four species, but the interaction between species and experiments was also significant. The proportions of all other amino acids on the leaf surface did not vary significantly between the experiments and between the four species. Consequently, it was impossible to discriminate between the four species on the basis of the proportions of a single amino acid. We therefore compared the four species with a discriminant function analysis. This analysis was performed with all data of the two experiments in order to increase the number of observations. All data were arcsine-root transformed. Methionine and tryptophan were left out of the analysis because their concentrations were too low. As the proportions of amino acids are not independent, we also left the most abundant amino acid (serine) out of the analysis, which diminishes this statistical problem. The discrimination between the species was perfect: either using the concentrations or the proportions of amino acids, we could ascribe 100% of the observations to the correct species (Figure 1). The amino acids that clearly discriminated between the four species were histidine on the first discriminant function; aspartine, threonine, glutamine, and glutamic acid on the second function, and GABA,

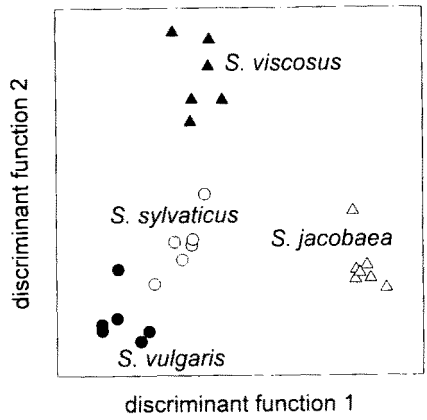

FIG. 1. Discrimination of four *Senecio* species on the basis of their proportions of amino acids on the leaf surface. Plot of all observations on the first two discriminant functions. The first and second discriminant functions contain 77.2 and 15.9 of the variation, respectively.

proline, alanine, phenylalanine, glycine, and tyrosine on the third function. In a discriminant function analysis using just these 11 amino acids, all observations were also classified correctly.

Amino Acids in Leaf Tissues of S. jacobaea

The mean total concentration of amino acids inside the leaf tissues of *S. jacobaea* in the four replicates of experiment 1 was 2.61 ± 0.11 mmol/m^2. The proportions of individual amino acids were very stable, as indicated by the small standard errors (Table 1). For some amino acids these proportions were very different from their proportions on the leaf surface. Inside the leaves, the dominant acids were serine, glutamic acid, aspartic acid, glutamine, and alanine, but the latter three were much less abundant on the leaf surface. Other amino acids, such as glycine, showed very low proportions inside the leaves but high proportions on the leaf surface. The correlation between the proportions of amino acids inside the leaf tissues and their proportions on the leaf surface, averaged over the four replicates (between the first two columns in Table 2), was consequently low: $r = 0.542$ ($N = 21$, $P = 0.03$).

Alkaloids on Leaf Surface of S. jacobaea

No alkaloids could be detected in any washing of the leaf surface of *S. jacobaea*. Taking into account the sensitivity of the alkaloid analysis, this means that if alkaloids were present on the leaf surface, their concentrations

were below 60 nmol/m^2. A second analysis on a large quantity of two of the samples showed that in these samples the concentrations of alkaloids, if present, must have been below 10.5 and 7.2 nmol/m^2, respectively. This, may still be above the detection limit of insects, however.

DISCUSSION

Our results show that the composition of free amino acids on the leaf surface of the four *Senecio* species is species-specific. It is generally believed that many phytophagous insects can perceive amino acids (Blaney and Simmonds, 1990; Schoonhoven and Blom, 1988). It is reasonable to suggest that such common substances that are so closely related to the nutritional value of a plant can be perceived by females seeking food plants for their larvae. This is supported by the results of another study, in which we found a behavioral response of ovipositing *Ostrinia nubilalis* to primary metabolites on the leaf surface of leek and maize (Derridj et al., 1996). The next important step in our investigation will be to determine responses of *Tyria jacobaeae* to amino acids and leaf surface washings, both sensorially and behaviorally.

The remarkable differences between the proportions of amino acids inside the leaf tissues and those on the leaf surface may reflect the selective passage of amino acids through the cuticle, caused by the physical and chemical properties of both the amino acids and the cuticle. As the composition and, thus, the physical and chemical properties of the cuticle and its intra- and epicuticular waxes varies between plant species (Jeffree, 1986; Espelie et al., 1991), it may be expected that the concentration of chemicals on the leaf surface also varies from species to species, as we show here. It must be stated, however, that passage through the cuticle can only be studied *in vivo* by comparing the proportions on the leaf surface to those in the apoplast. Passage through this free space (the cell wall volume between adjacent cells) is obligatory for amino acids to reach the leaf surface. The amino acid composition of the leaf apoplast is probably quite different from that which we determined for the whole leaf.

Many factors may change the concentrations of free amino acids on the leaf surface where they are directly exposed to the environment. Rain, dew, and fog may leach the amino acids from the leaf (Tukey, 1971), but also epiphytic microorganisms may modify the biochemical composition of the leaf surface (Schreiber and Schönherr, 1992), in particular with respect to amino acids (Martin et al., 1993). We know nothing at present about the mechanisms by which the proportions of free amino acids on the leaf surface are stabilized.

As the passage through the cuticle is probably passive, it is unlikely that large molecules like pyrrolizidine alkaloids (molecular weight 300–400) can leave the inner tissues, which explains why we did not find them on the leaf

surface of *S. jacobaea*. Although in some plant species alkaloids were found on the leaf surface (Tukey, 1971), they are probably usually stored in the vacuoles, as found by Ehmke et al. (1988) in *S. vulgaris*. In that case, the way for *Tyria* to detect alkaloid-containing plants could be by detecting the presence of smaller precursors, such as, amino acids or polyamines on the leaf surface. Such indirect assessment of secondary metabolites may be common among phytophagous insects and complicates investigation of the substances responsible for host recognition.

We do have preliminary information that the proportions of organic acids on the leaf surface of the *Senecio* species studied here are also more or less species-specific. The same may be true for numerous other substances that are present on the leaf surface after leaking through the cuticle from the inner tissues. Together, all these substances may constitute a distinctive chemical image of the plant, enabling phytophagous insects to select between different plant species. Secondary chemicals are just a part of this mixture, which may explain why it is so difficult to find a single secondary chemical that explains host specificity of an insect (Jermy, 1993; Renwick and Chew, 1994). To conclude: "Errors of judgement . . . are only avoidable by a careful examination and comparison of many types of characters" (Hegnauer, 1986), and the problem of recognizing a plant species may be the same for phytophagous insects and chemotaxonomists.

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CHEMOSENSORY PREDATOR RECOGNITION IN THE LIZARD *Podarcis hispanica:* EFFECTS OF PREDATION PRESSURE RELAXATION

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Abstract—We compared the behavior of two subspecies of *Podarcis hispanica* lizards in cages that had been chemically marked by a saurophagous snake, the viper *Vipera latastei*. One of the subspecies (*P.h. atrata*) has experienced a relaxation from predation pressure by this viper, as snakes were eradicated from the island it inhabits over 100 years ago. Nevertheless, individuals from *P.h. atrata* responded to the snake's chemicals similarly to individuals from a population of *P.h. hispanica*, currently sympatric with *V. latastei*. Lizards exhibited more stress-indicating behaviors (foot shakes, tail vibrations, sudden starts), became less mobile, and tongue-flicked more while moving in a snake-inhabited terrarium than when in a clean, unfamiliar terrarium.

Key Words—Antipredator behavior, chemoreception, lizards, Lacertidae, Reptilia, predation, relaxation, snakes.

INTRODUCTION

Predation is an important selective force that can shape the morphological, behavioral, and life-history characteristics of prey animals. Studies on a variety of species [e.g., spiders (Riechert and Hedrick, 1990), fish (Magurran and Seghers, 1990; Gelowitz et al., 1993), salamanders (Dowdy and Brodie, 1989; Ducey and Brodie, 1991), snakes (Sweet, 1985), and mammals (Owings and Coss, 1977)] have demonstrated that geographical variation in predation pressure is reflected in parallel variation in antipredator behavior of prey animals; prey animals from predator-free or -poor habitats fail to recognize predators in staged

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encounters (Magurran, 1986; Magurran and Seghers, 1990; Gelowitz et al., 1993; Mathis and Smith, 1992) or show inappropriate responses to predator stimuli (Breden et al., 1987; Dowdey and Brodie, 1989; Herzog and Schwartz, 1990; Riechert and Hedrick, 1990). These intraspecific differences in antipredator behavior may arise from differential experience within the lifetime of an individual (Magurran, 1989; Olla and Davis, 1989; Jaervi and Uglem, 1993), from natural selection acting upon genetic variation (Huntingford, 1982; Breden et al., 1987; Riechert and Hedrick, 1990), or from a combination of both (Giles and Huntingford, 1984; Magurran, 1990).

In this paper, we compare the ability of two subspecies of the lacertid lizard *Podarcis hispanica* to detect chemicals from the saurophagous snake *Vipera latastei*. Predator recognition is a necessary first step of adequate antipredator behavior. The vomeronasal system of reptiles offers good opportunity to study the ability to recognize predatory cues, without the need to actually confront the animals with the predator itself. Reptiles have been shown to react to chemicals left behind by predators with an increased rate of tongue extrusions (Chiszar et al., 1978; Thoen et al., 1986; Van Damme et al., 1990, 1995). The intensified movements of the forked tongue probably facilitate delivery of chemical substances to the ducts leading to Jacobson's organ (Parsons, 1970). Tongue-flick rate can therefore be used as a quantitative bioassay of interest in a variety of stimuli, including food and predator chemical cues.

One of the lizard subspecies under study, *P.h. atrata*, is endemic to the Mediterranean archipelago of the Columbretes Islands (Castellón, Spain). A large population of *Vipera latastei* inhabited the main island of this archipelago but was completely eradicated when a lighthouse was constructed by the end of the 19th century. In spite of several herpetological expeditions, not a single live snake was observed after 1886 (Castilla et al., 1987). The other subspecies, *P.h. hispanica*, lives on the nearby mainland of Spain (ca. 50 km away) and is still sympatric with *V. latastei*. Our objective was to test whether the recent relaxation of the predation pressure in *P.h. atrata* has resulted in the loss of the ability to recognize viper chemicals. If *P.h. atrata* still reacts to the snake chemicals, this strongly suggests that the chemosensory recognition of predator cues is genetically based, since none of the individuals has ever been in contact with snake predators. Alternatively, lizards may respond to chemicals that the viper has in common with other predators now present on the island. If *P.h. atrata* fails to recognise the stimuli, recognition is either based on experience and/or has been lost in the island population.

METHODS AND MATERIALS

Typically, *Podarcis hispanica* is a small [snout-vent length 40–60 mm], slender, flattened wall lizard living on rocks, stony slopes, walls, and other

habitats dominated by vertical elements. Like most other lacertids, *P. hispanica* has an active-searching foraging mode, a predominantly arthropod-based diet, and a diurnal activity pattern. It should be noted, however, that the species exhibits substantial variation in body dimensions and habitat preferences throughout its distributional range in Iberia and northwest Africa (Salvador, 1986). The population of *P.h. hispanica* sampled here has the typical characteristics described above. These lizards lived on garden walls and in a small park and were almost exclusively seen on stony substrates elevated more than 0.5 m above ground. Adult SVL ranged between 40 and 55 mm ($\bar{X}$ = 46.9 mm; SD = 3.7 mm; N = 27). The subspecies *P. hispanica atrata*, from Columbretes Islands, is aberrant in its large adult body size (range 52–71 mm; $\bar{X}$ = 60.5 mm; SD = 4.2 mm; N = 44). Although lizards from this population climb regularly, they are most frequently seen on flat, horizontal surfaces, often at ground level.

Vipera latastei is a rather small (adult SVL 50–60 cm), stocky snake from the Iberian Peninsula and northwest Africa. It prefers sunny, stony, and slightly vegetated slopes and open deciduous forest. The snake feeds on rodents, lizards, and bird nestlings, killing its prey by injecting neurotoxic and hemorraghic venom (Gruber, 1989).

We caught 10 adult *Podarcis hispanica atrata* (four males and six females) on the bigger island of the Columbretes archipelago (Columbrete Grande) and 10 *P.h. hispanica* (six males and four females) from the nearby mainland (Castellón, Spain) and transported them to the laboratory in Antwerp, Belgium. Lizards were housed in five large terraria and daily were given ample amounts of food (crickets and mealworms) and water. Two 150-W spotlights suspended above both extremes of each terrarium provided light and heat for 10 hr/day. Individuals from both populations were kept apart. A male viper (*Vipera latastei*, SVL 45 cm) was obtained from the province of Burgos (Spain) and was kept in a cage located in a separate room. We took care that none of the lizards was in visual or chemical contact with this viper outside experimentation.

We followed the experimental procedure outlined in Thoen et al. (1986) and Van Damme et al. (1990) to assess the lizards' responses to snake chemical cues. We introduced each individual lizard successively into two small terraria. These terraria were identical in dimensions (25 × 25 cm), substrate (sand, mosses, and stones), illumination, and heating (provided by a 150-W spotlight suspended 22 cm above the substrate). One of the cages (hereafter referred to as the viper situation) had previously been inhabited by the viper. After the snake had resided in the cage for at least 12 hr, we removed it and replaced it by a lizard. The other test terrarium was free from any viper chemicals; we used it to monitor lizard behavior in an unfamiliar but predator-free situation. The order in which a lizard was tested in the control and the viper situation was decided by chance. Since *P. hispanica* is known to react to chemicals left behind

on the substrate by conspecifics (Gómez et al., 1993), we changed the sand and vegetation and rinsed the stones from the testboxes after each experiment.

We observed the lizards' behavior from behind a one-way mirror. Observations started about 10 sec after transfer of the lizard to the test box and lasted 15 min. We distinguished the following behavioral acts and locomotor patterns. (1) Tongue-flick: the lizard extrudes and rapidly retracts its tongue, regardless of whether the tongue touches the substrate or is "waved" in the air. (2) Walk: continuous, relatively fast forward movement. This is the locomotor type typically observed in unrestrained lizards. (3) Slow motion: very slow movement accompanied by jerky or waving movements of the forelimbs. (4) Stand up: the lizard stands in an upright position against the glass wall of the terrarium and performs scratching movements with the forelegs. (5) No move: the lizard stands still, its ventrum resting on the substrate; this pattern can be accompanied by movements of head, tail or forelimbs. (6) Bask: the lizard rests under the light bulb with the ribs spread laterally; one or more feet are often tilted upwards. (7) Start: sudden jump, most often followed by a quick, short run. (8) Foot shake: the raised forelimbs are alternately and rapidly moved up and down. (9) Tail vibration: the entire tail, or its posterior portion, is moved rapidly from side to side.

We used a BASIC program run on a personal computer to record the lizards' behavior. The program assigns a key to each of the behavioral acts distinguished and checks every second which key on the computer keyboard is being pressed by the observer. At the end of an observation period, the program lists for every second the behavior performed by the lizard, the number of tongue flicks, starts, foot shakes, and tail vibrations.

We used 2×2 factorial repeated measures analysis of variance on the total duration or frequency of behavioral acts to test for differences between treatments (viper/control) and populations. Lizards clearly extruded their tongues more frequently while moving than when basking or resting. Therefore, if we want to use tongue-flick frequency as a bioassay for predator recognition, we have to correct for the amount of time the lizard spent moving. We did this by introducing the total duration of walk and slow motion as a covariate into the analysis of variance.

Lizard activity typically consists of alternating mobile (foraging, exploration) bouts and stationary periods (basking, resting). We suspected that the length and frequency of these bouts might differ between treatments and/or populations. There is a practical difficulty in delimiting a locomotion bout, because lizards in general do not move in a continuous way, but now and then pause for a couple of seconds before resuming their itinerary. We used an algorithm described by Sibly et al. (1990) to distinguish between these short stops within a locomotion bout and "real" stationary periods. In this method, two regression models (a one-process model and a two-process model) are fitted

to a $\log_e$ frequency distribution of gap lengths. A gap is every interval between two successive movements. The one-process model assumes that all events are generated by a single Poisson process, and thus that behavior cannot be split into bouts. The two-process model considers events to be generated by one of two random Poisson processes, a fast process operating within bouts and a slow process generating new bouts. In our case, the two-process model clearly fitted better to the data than the one-process model. We derived a bout-criterion interval (Slater and Lester, 1982) of 28 sec, which means that gaps between real locomotory bouts must at least be this long. If the interval is shorter than 28 sec, the gap is considered as a short pause within a movement. Using this criterion, we calculated for each observational period the average locomotory bout length and frequency, the length of the interval between bouts, and the number and frequency of pauses within locomotion bouts. These new behavioral parameters were compared between treatments and populations using repeated-measures analysis of variance. Since the number of pauses is clearly dependent on the time spent moving, we corrected for this by introducing total bout duration (mean bout length × frequency) as a covariate into the analysis. All statistical analyses were performed with the SPSS/PC+ statistical package (Norusis, 1990).

RESULTS AND DISCUSSION

Lizards of both populations reacted to the former presence of *Vipera latastei* in the test terrarium by an increased number of starts, foot shakes, and tail vibrations (Table 1). These behaviors are typically displayed by lizards in conflict situations, during encounters with conspecifics (Verbeek, 1972), potential prey, or predators (Nuyten and Van Wezel, 1984; Thoen et al., 1986; Van Damme et al., 1990, 1995). Since the presence or absence of predator chemicals constitutes the only difference between our two test situations, we assume that the increase in these stress-indicative behaviors in the viper situation represents a shift in motivational status towards enhanced awareness and possibly fear.

We found significant treatment × population interaction effects for the number of foot shakes and tail vibrations. This suggests that although lizards from both populations respond to viper chemicals, the predator-sympatric *P. hispanica hispanica* reacted more vigorously than the predator-allopatric *P. hispanica atrata*. This differentiation was, however, not evident in the other stress-indicative behavioral components (the number of tongue-flicks and starts).

In contrast to *Lacerta vivipara* (Thoen et al., 1986; Van Damme et al., 1990, 1995), *Podarcis hispanica* did not tongue-flick more in the viper situation than in the control situation. This finding is deceptive, however, because tongue-flick frequency is clearly higher during locomotion, and lizards moved significantly less in the viper situation than in the control situation. After correction

TABLE 1. BEHAVIOR OF LIZARDS FROM POPULATIONS ALLOPATRIC (COLUMBRETES) AND SYMPATRIC (CASTELLÓN) WITH *Vipera latastei*[a]

	P.h. atrata Columbretes		*P.h. hispanica* Castellón		*P*		
Variable	Control	Viper	Control	Viper	Treat	Pop	Pop × treat
Walk	461.4 ± 142.4	193.9 ± 172.8	248.0 ± 198.0	205.2 ± 146.5	0.009	0.03	0.004
Slow motion	7.8 ± 9.9	4.0 ± 9.8	58.5 ± 84.9	4.3 ± 5.1	0.07	0.05	0.07
Bask	56.7 ± 29.0	92.0 ± 69.8	110.6 ± 97.9	101.3 ± 47.5	0.16	0.53	0.31
No move	310.1 ± 143.8	587.7 ± 260.6	436.8 ± 253.0	557.8 ± 182.6	0.22	0.04	0.05
Stand up	50.1 ± 42.0	3.7 ± 3.6	21.8 ± 33.1	15.4 ± 17.6	0.31	0.01	0.02
Dig	13.9 ± 17.5	18.7 ± 35.9	24.3 ± 32.5	16.0 ± 27.6	0.35	0.87	0.58
Tongue flicks	226.1 ± 45.1	162.6 ± 124.1	277.2 ± 117.8	220.1 ± 110.9	0.04	0.15	0.90
Starts	0.6 ± 1.07	0.9 ± 1.5	2.8 ± 2.0	3.5 ± 4.8	0.015	0.82	0.58
Foot shakes	0.1 ± 0.3	0.3 ± 0.6	0.2 ± 0.4	4.3 ± 5.0	0.02	0.02	0.02
Tail vibrations	0.1 ± 0.3	0.3 ± 0.7	0.2 ± 0.4	4.3 ± 5.0	0.02	0.02	0.02
Locomotory bouts	2.8 ± 1.5	5.7 ± 3.6	4.6 ± 2.9	5.7 ± 1.5	0.02	0.32	0.18
Bout length	394.7 ± 279.7	174.5 ± 149.6	90.5 ± 127.3	59.9 ± 44.8	0.03	0.003	0.06
Gap length	57.1 ± 27.7	51.8 ± 16.3	75.3 ± 39.8	96.3 ± 59.5	0.56	0.14	0.27
Stops	44.6 ± 12.2	29.9 ± 16.2	18.1 ± 16.1	17.4 ± 14.0	0.09	0.002	0.05
Stop length	6.9 ± 2.4	8.6 ± 2.4	8.3 ± 1.5	10.4 ± 4.5	0.24	0.20	0.45

[a]Lizards were observed in a terrarium that had been previously inhabited by a snake (viper) and in an unfamiliar, but clean, terrarium (control). Presented are the number or duration (seconds) of behavioral acts observed during 15-min observation periods. Values are means (±SD, $N = 10$ for both populations). The last three columns give the P values for the effects of population, treatment, and the interaction between population and treatment, as derived from repeated-measures analysis of variance.

for the amount of time spent moving (walk + slow motion), lizards of both populations extruded their tongues more often in the viper situation than in the control terrarium (Figure 1, $F = 8.38$, $df = 1$ and 17, $P = 0.01$). This finding suggests that lizards perceive chemical clues indicating the presence of the viper and that these stimuli invoke an enhanced utilization of the chemoreceptive apparatus.

We found no population or treatment × population interaction effects for the corrected tongue-flick frequency (both $P > 0.2$). We conclude that the ability to detect viper chemicals must be genetically based in *P.h. atrata*, since none of the lizards from this subspecies had any experience with predatory snakes before. Furthermore, 100 years of relaxed predation pressure have not resulted in the loss of the ability for chemoreceptive recognition. Alternatively, lizards may respond to chemicals that the viper has in common with other predators present in its habitat. Little is known regarding the degree of specificity of responses by the lizard to such chemicals. In the case of *P.h. atrata*, however, this alternative explanation seems unlikely, since the Columbretes Islands are

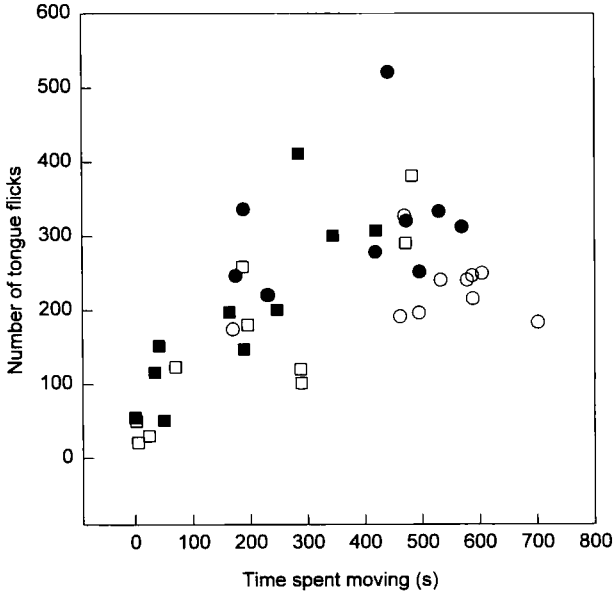

FIG. 1. Number of tongue flicks performed by the lizard *Podarcis hispanica* as a function of the time spent moving. Lizards were observed for 15 min in terraria previously inhabited by a viper (filled symbols) and in unfamiliar but clean terraria (open symbols). Circles represent individual lizards from a predator-allopatric population (*P.h. atrata*), and squares refer to lizards from a predator-sympatric population (*P.h. hispanica*).

currently nearly devoid of potential predators. There are no snakes or mammals and no typical saurophagous birds such as corvids or shrikes (Castilla et al., 1987), leaving only two species of gulls (*Larus cachinnans michahellis* and *Larus audouinnii*) as potential predators of lizards. These gulls primarily predate or scavenge on fish and marine invertebrates (Cramp, 1983), but it is not impossible that they occasionally attack a lizard.

Locomotion patterns differed between populations and treatments. In the control situation, *P.h. atrata* showed a much higher tendency to explore the cage than *P.h. hispanica* and thus obtained higher mobility scores (Table 1). This difference in mobility between populations was largely due to the longer mean duration of locomotion bouts performed by the island subspecies (Table 1). Although we cannot present quantitative data to support this, the observed differences in locomotory activity seem to persist outside our experimental situations. Both in the field and in their home cages in the laboratory, individuals of *P.h. atrata* can be seen moving about far more often than their conspecifics from the mainland. Although it is tempting to ascribe this difference to the higher predation risk on the mainland, many other factors involved in the cost-benefit balance of locomotory activity (e.g., body dimensions, prey density, social context, etc.) may also be involved.

Locomotion in *P. hispanica* is not smooth and continuous, but consists of a pattern of bursts of movement alternating with pauses. Although seemingly widespread among lizard species (see Avery et al., 1987, and references therein), the exact ecological significance and/or the physiological basis of this jerky motion pattern remains obscure. In our experiments, the number of pauses per unit time traveled and the mean duration of pauses did not differ between species or treatments. Therefore, we have no reasons to suppose that the frequent stops are a part of the antipredator behavior of *P. hispanica*.

We conclude that the lizard *P. hispanica* is capable of detecting the former presence of a predatory snake, the viper *V. latastei*, presumably through chemosensory perception of chemicals left behind on the substrate. The ability is still present in individuals from a population that has experienced relaxation from predation by snakes during the past 100 years. In *P.h. atrata*, the response to the viper's former presence may constitute another example of a "ghost of predation past" (Peckarsky and Penton, 1988; cf. Connell, 1980) or may reflect responses to chemicals common to a range of predatory species. Assuming that the ability to detect predator chemicals is genetically based, it may not be surprising that it has survived in the viper-free population. The rate at which vestigial characters disappear from a population is likely to be a negative function of the costs of expressing the character, and we cannot think of any substantial costs involved in this case. It would be interesting to study predator recognition abilities in populations that are liberated from specific predators for longer periods of time.

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INVESTIGATION OF THE ROLE OF DITERPENES PRODUCED BY MARINE PULMONATES *Trimusculus reticulatus* AND *T. conica*

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Abstract—The intertidal pulmonate limpet *Trimusculus reticulatus*, which is found in caves or crevices along the California coast, was previously reported to contain two novel diterpenoids, 6β-isovaleroxylabda-8,13-dien-7α,15-diol (**1**) and 2α,7α-diacetoxy-6β-isovaleroxylabda-8,13-dien-15-ol (**2**). Dissection of the animals prior to extraction revealed that the diterpenoids were concentrated in the mantle, foot, and mucus, but not in the viscera. The presence of *T. reticulatus* or its mucus was toxic to veliger larvae of the sabellariid reef-building tube worm *Phragmatopoma californica*. The major diterpenoid **1** was responsible for the observed larvicidal activity. Protection against overgrowth by settling invertebrate larvae is important for the survival of this sessile filter-feeding pulmonate. A related pulmonate, *T. conica*, which was found in similar intertidal habitats in New Zealand, contained 6β-acetoxy-7α-isovaleroxylabda-8,13-dien-15-oic acid (**3**) and 1β,7β,12β,18-tetraacetoxycholest-5-en-3β-ol (**4**). The diterpene **3** was also localized in the foot, mantle, and mucus of *T. conica* but was not larvicidal to *P. californica*.

Key Words—*Trimusculus reticulatus*, *Trimusculus conica*, pulmonate, limpet, sessile, filter feeding, mucus, secondary metabolite, diterpene, steroid, *Phragmatopoma californica*, larvicidal.

INTRODUCTION

The intertidal pulmonate limpets of the family Trimusculidae are unusual in both their habitat and behavior. The air-breathing trimusculids, which lack a gill and

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instead have a modified mantle cavity that serves as a lung, live on exposed rocky coastlines and are found only in narrow crevices and deep caves where they hang inverted and tightly clustered in large colonies (Walsby et al., 1973). The adults are essentially sessile, and fertilization is internal in these hermaphrodites. The eggs are brooded under the shell on each side of the foot and, after direct development, the young emerge from the eggs as crawling slugs (Haven, 1973). Unlike most intertidal limpets, which feed by grazing on thinly encrusting microalgae, trimusculids filter feed by producing a mucous net that traps suspended phytoplankton from the surging tide. The limpet uses its oral lobes to inspect the mucous net frequently for the presence of food and then the mucus and trapped particles are ingested (Walsby et al., 1973). An abundance of subepithelial glands on the mantle and sides of the foot produce the copious milky white mucus, which has been suggested to be repugnatorial (Yonge, 1958). Mucus collected from *T. reticulatus*, which occurs on the California coast, was shown to repel the predatory seastars *Pisaster ochraceus* and *P. giganteus* (Rice, 1985).

Due to their unique lifestyle, the trimusculids live with unusual constraints. They are essentially sessile and do not exhibit the common flight response that most limpets display when in the presence of a seastar. Additionally, their method of collecting food by filter feeding necessitates that they be undisturbed by other encrusting invertebrates that could occupy this space in the intertidal zone. Our earlier report of relatively large quantities of diol **1** and diacetate **2** (Figure 1) isolated from *Trimusculus reticulatus* and its mucus (Manker and Faulkner, 1987) raised questions concerning the possible role the chemicals may play in fulfilling the unusual ecological needs for these pulmonate limpets.

Many marine molluscs have been shown to contain secondary metabolites, and there have been numerous studies investigating the role of these compounds. The dorid nudibranchs are a shell-less order of gastropods that have been shown, in some cases, to sequester secondary metabolites from their specialized sponge diet. These animals have few predators despite their lack of a shell or other means of physical defense. In addition, many are brightly colored, and this has often been thought to have an aposematic function. Several studies have been carried out to determine the ecological role of storage of sponge metabolites in nudibranchs (Thompson et al., 1982; Hagadone et al., 1979; Castiello et al., 1980; reviewed by Faulkner, 1992), and these cases support the hypothesis that the compounds are used as a chemical defense to deter predation. A correlation between the lack of a shell and alternative defense mechanisms has been suggested by Faulkner and Ghiselin (1983). In addition, a number of molluscs bearing shells have also been shown to contain unusual secondary metabolites. The shell-bearing archeaogastropod limpet *Collisella limatula* was found to contain a defensive triterpene, limatulone **5** (Figure 1) (Albizati et al., 1985). Pawlik et al. (1986) reported that intertidal fish and crabs rejected pieces of the foot of

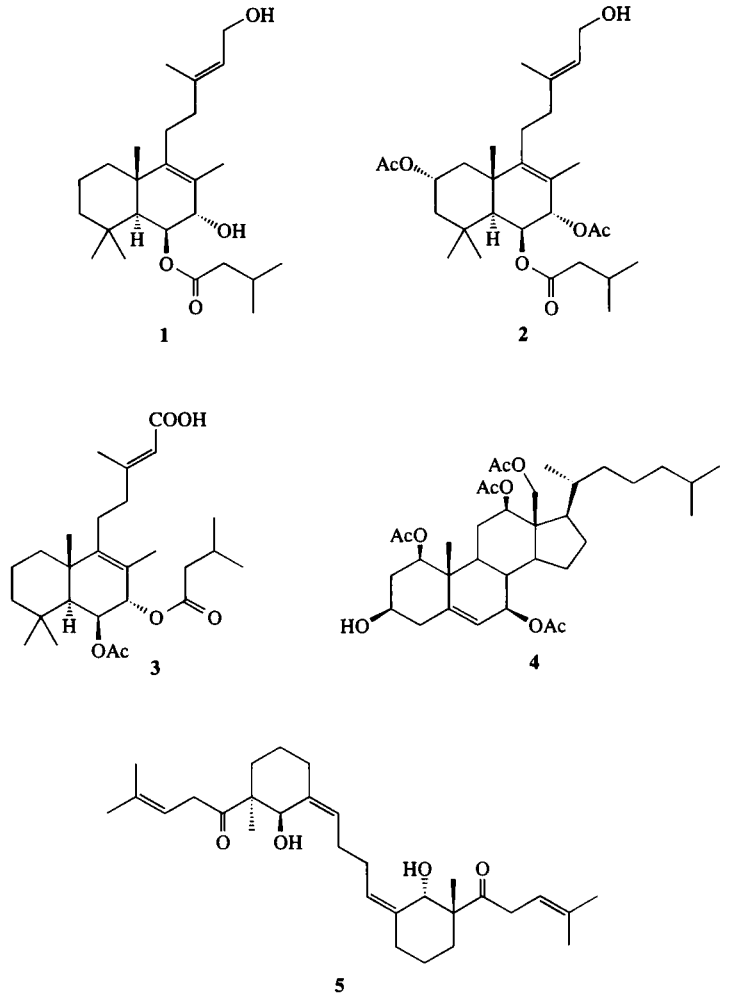

FIG. 1. Structures of compounds **1** and **2** found in *Trimusculus reticulatus*, compounds **3** and **4** found in *T. conica*, and compound **5** from *Collisella limatula*.

C. limatula but consumed foot tissue of other gastropods that occur in the same area. Food pellets treated with natural concentrations of limatulone caused regurgitation in the intertidal fish *Gibbonsia elegans*, a known limpet predator. Pulmonate limpets of the family Siphonaridae, which are closely related to the trimusculids, contain a plethora of secondary metabolites. However, a role for these compounds has not been established (Manker, 1986).

Considering the unusual habitat (Haddock, 1989) and life-style of the trimusculids, competition for space may be a high priority. One of the most important resources in the intertidal zone is space, for which competition is often intense. Many intertidal invertebrates have planktonic larvae that settle on a suitable substrate before metamorphosis into the juvenile form. A plausible strategy for these sessile, intertidal limpets might be to produce chemicals that are toxic to settling larvae in order to keep the area around them free of other invertebrates. This antifouling function has been invoked for sponges that often contain antimicrobial metabolites and that need to keep their surfaces clean to be efficient filter feeders (Thompson et al., 1985). However, fouling would be just as important in the intertidal community. To date, no relevant research has been done on the pulmonates in this area.

In this paper, we report the results of investigations into the distribution and role of secondary metabolites from trimusculid limpets.

METHODS AND MATERIALS

General. ^{1}H NMR spectra were recorded on 360 MHz Nicolet or Bruker WP-200 SY spectrometers. ^{13}C NMR spectra were run at 50 MHz on the Bruker WP-200 SY spectrometer. High-resolution mass spectra were obtained from the Regional Mass Spectrometry Facility at U.C. Riverside. High-performance liquid chromatography separations were carried out using a Waters model 6000A solvent delivery system monitored by a Waters model 440 absorbance detector and a Waters model R401 refractive index detector. All solvents used were either HPLC grade or distilled in glass prior to use.

Collection and Extraction of Trimusculus reticulatus. The animals (N = 115) were collected intertidally from crevices between boulders on San Nicholas Island, California. Mucus produced by the specimens was separated and lyophilized, then extracted with ether. Whole animals were lyophilized and extracted sequentially with hexane, dichloromethane, and ethyl acetate. The dichloromethane extract was separated by HPLC on silica, using 9:9:2 hexane–ethyl acetate–isopropanol as eluant, followed by HPLC using 45% hexane in ethyl acetate as eluant to obtain 6β-isovaleroxylabda-8,13-dien-7α,15-diol (**1,** 201 mg) and 2α,7α-diacetoxy-6β-isovaleroxylabda-8,13-dien-15-9-ol (**2,** 4 mg). The extracted mucus was purified in the same manner to obtain the pure diol (**1,** 9 mg, 78 μg/animal).

Dissection of T. reticulatus. Seven individuals of *T. reticulatus* were lyophilized and then dissected to separate the foot, mantle, viscera, and shell. The dissected components were separately soaked in acetone for 12 hr, after which time the acetone was decanted and evaporated to dryness. The crude organic extracts of each component were analyzed by ^{1}H NMR spectroscopy. Each crude organic extract was further purified by HPLC to obtain individual compounds.

Collection and Extraction of Trimusculus conica. The animals (N = 123) were collected intertidally from cracks between boulders at Anawhata Cove on the North Island of New Zealand. The animals were stored in acetone for one week, and then the solvent was decanted and evaporated to obtain an aqueous suspension. The aqueous phase was extracted with ethyl acetate (4 × 50 ml), and the extracts were combined, dried over anhydrous sodium sulfate, and evaporated to obtain a brown oil (405 mg). The crude extract was purified by flash chromatography on silica using 1:1 hexane–ethyl acetate as eluant. The fractions containing the UV active material were combined and applied to a 1000-μm preparative silica gel TLC plate and developed in 2:3 hexane–ethyl acetate. The UV active band was removed from the silica gel plate, and the silica was extracted with ethyl acetate to yield 6β-acetoxy-7α-isovaleroxylabda-8,13-dien-15-oic acid (**3,** 75 mg) (Figure 1). A second less polar UV active band was also removed and extracted to obtain a yellow oil (21 mg). The oil was purified by HPLC on partisil using 3:7 hexane-ethyl acetate as eluant to obtain 1β,7β,12β,18-tetraacetoxycholest-5-en-3β-ol (**4,** 12 mg).

6β-Acetoxy-7α-isovaleroxylada-8,13-dien-15-oic Acid (3). Oil; $[\alpha]_D$ = +64.1 (*c* 1.13, $CHCl_3$); IR ($CHCl_3$) 2950, 1740, 1690, 1640 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.96 (s, 3H), 0.97 (s, 3H), 0.98 (d, 6H, J = 6 Hz, H-24, H-25), 1.26 (m, 1H), 1.30 (s, 3H, H-18), 1.4 (m, 1H), 1.5 (m, 1H), 1.6 (s, 3H, H-17), 1.8 (br d, 1H, J = 13 Hz, H-5), 2.05 (s, 3H, 6-OAc), 2.1 (m, 1H), 2.22 (s, 3H, H-16), 2.25 (m, 2H), 4.99 (br s, 1H, H-7), 5.33 (br s, 1H, H-6), 5.74 (br s, 1H), H-15); ^{13}C NMR ($CDCl_3$) δ 16.9 (q), 18.8 (t), 19.1 (q), 20.9 (q), 21.3 (q, C-18), 22.2 (q, C-24), 22.3 (q, C-25), 22.9 (q, C-19), 25.8 (d, C-23), 26.4 (t, C-11), 32.9 (q, C-20), 33.4 (s, C-4), 38.9 (s, C-10), 39.4 (t, C-1), 40.8 (t, C-12), 43.0 (t, C-3), 43.5 (t, C-22), 49.1 (d, C-5), 69.5 (d, C-6), 72.0 (d, C-7), 114.9 (d, C-14), 122.3 (s, C-8), 146.7 (s, C-9), 162.2 (s, C-13), 169.6 (s, C-15), 171.8 (s, C-26), 172.0 (s, C-21); EI-MS, Obs. m/z = 445.2962, $C_{27}H_{41}O_5$ $(M\text{-}OH)^+$ requires m/z = 445.2954.

1β,7β,12β,18-Tetraacetoxycholest-5-en-3β-ol (4). Oil; $[\alpha]_D$ = +29.7 (*c* 1.13, $CHCl_3$); IR ($CHCl_3$) 3500 cm^{-1} (br), 1730 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.86 (d, 3H, J = 6.5 Hz, H-26), 0.87 (d, 3H, J = 6.5 Hz, H-27), 0.92 (d, 3H, J = 7.2 Hz, H-21), 1.09 (s, 3H, H-19), 2.03 (s, 3H, OAc), 2.04, (s, 6H, OAc), 2.10 (s, 3H, OAc), 2.25 (m, 2H), 2.35 (m, 2H), 3.55 (m, 1H, H-3), 4.27 (d, 1H, J = 12.4 Hz, H-18), 4.35 (d, 1H, J = 12.4 Hz, H-18), 4.68 (dd, 1H, J = 11.2, 5.1 Hz, H-12), 4.90 (br d, 1H, J = 10.9 Hz, H-1), 5.00 (br d, 1H, J = 8.6 Hz, H-7), 5.22 (br s, 1H, H-6); ^1H NMR (C_6D_6) δ 0.80 (s, 3H, H-19), 0.92 (d, 3H, J = 6.5 Hz, H-26), 0.94 (d, 3H, J = 6.5 Hz, H-27), 1.11 (d, 3H, J = 6.9 Hz, H-21), 1.56 (m, 3H), 1.73 (s, 3H, OAc), 1.76 (s, 3H, OAc), 1.77 (s, 3H, OAc), 1.81 (s, 3H, OAc), 1.91 (m, 2H), 2.10 (bd, 1H), 2.14 (m, 3H), 3.27 (tt, 1H, J = 12, 4 Hz, H-3), 4.43 (d, 1H, J = 12.3 Hz, H-18), 4.56 (d, 1H, J = 12.3 Hz, H-18), 4.70, (dd, 1H, J = 11.2, 4.7 Hz,

H-12), 5.14 (br d, 1H, J = 8.7 Hz, H-7), 5.21 (br d, 1H, J = 11 Hz, H-1), 5.39 (br s, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 14.1 (q), 18.9 (q), 21.3 (2xq), 21.5 (q), 21.7 (q), 22.4 (q), 22.7 (q), 24.7 (t), 25.0 (t), 27.8 (d), 28.5 (t), 28.7 (t), 31.2 (t), 35.6 (d, t), 36.4 (s), 36.6 (t), 37.9 (d), 41.3 (t), 47.2 (d), 49.5 (s), 53.5 (d), 54.0 (d), 61.4 (t, C-18), 70.9 (d), 74.9 (d), 76.9 (d), 79.2 (d), 121.1 (d, C-6), 145.1 (s, C-5), 170.2 (s), 170.7 (s), 170.9 (s), 171.0 (s); EIMS, Obs. m/z = 601.3748, $C_{35}H_{53}O_8$ $(M\text{-}OH)^+$ requires m/z = 601.3740.

Dissection of T. conica. Thirty-three specimens of *T. conica* were lyophilized and then dissected to separate the foot, mantle, viscera, and shell. The dissected components were separately soaked in acetone for 12 hr, after which time the acetone was decanted and evaporated to dryness. Each crude organic extract was analyzed by ^{1}H NMR spectroscopy and then subjected to HPLC to obtain pure compounds.

Seastar Response to T. reticulatus. One individual of *T. reticulatus* was placed in an aquarium containing one *Astrometis* sp., and the response was recorded for 30 min (three trials). In control experiments, the response of *Astrometis* sp. to the presence of *Colisella limatula* or *C. scabra* was recorded in a similar manner. In a separate experiment, mucus was collected from one individual of *T. reticulatus* or *C. limatula* by molesting the animals with a cotton swab until no further mucus production was observed. Each mucus-containing cotton swab was presented to the tip of the arm of an *Astrometis* sp. and the response recorded (three trials).

Fish Feeding Deterrence. Pure compounds were dissolved in ether or acetone and injected into freeze-dried krill pellets (*Euphausia* sp., San Francisco Bay Brand Ocean Plankton, 4–6 mg each) to reach a concentration of 100 μg compound/mg krill. Pellets were then dried under nitrogen. Control pellets were treated with solvent alone. Experimental and control pellets were paired and randomly fed to fish (*Thallasoma lunare*). The responses were recorded as eaten or, if the pellet was regurgitated three or more times, as rejected. The assay was repeated twice for each compound. The known repugnatorial compound limatulone (Pawlik et al., 1986) was used as a positive control.

Inhibition of Larval Settlement. Planktotrophic larvae of *Phragmatopoma californica* were reared at 20°C on a 1 : 1 mixture of *Phaeodactylum tricornutum* and *Monochrysis lutheri* under the same conditions described previously by Pawlik (1986). After 20–30 days, 10 larvae were transferred to 100-mm-diameter Petri dishes filled with 10 ml of 1-μm-filtered seawater. Each dish contained 1 g of Ottawa sand (20–30 mesh, Fisher Scientific), which had been treated in one of four ways: (1) control—100 μl ether; (2) settling inducer—100 μg palmitoleic acid in 100 μl ether; (3) settling deterrent—a known quantity of a pure compound in 100 μl of ether; or (4) larval toxicity—100 μg palmitoleic acid and a known quantity of a pure compound in 100 μl ether. After 24 hr, larvae were scored as swimming (not metamorphosed, no loss of provisional setae or

rotation of larval tentacles), metamorphosed (attachment, loss of provisional setae, rotation of larval tentacles or tube building), or dead.

For experiments with *Trimusculus reticulatus*, a whole limpet was placed in a Petri dish with 1 g of sand and 10 ml of filtered seawater. *Collisella* sp. were used as controls. In another treatment, mucus collected from one specimen of *T. reticulatus* was spread onto 1 g of sand and subsequently freeze-dried. Ten larvae were added and after 24 hr, larvae were scored as swimming, metamorphosed, or dead. Three replicates were carried out for each experiment.

RESULTS

Chemical Studies of T. conica. Investigation of the organic extract of this species resulted in the isolation of two novel compounds. 6β-Acetoxy-7α-isovaleroxylabda-8,13-dien-15-oic acid (**3**), $[\alpha]_D = +64.1$, was isolated as a colorless oil. The molecular formula, $C_{27}H_{42}O_6$, was deduced from ^{13}C DEPT experiments and from a fragment ion peak in the mass spectrum, $m/z = 445.2962$ $(M\text{-}OH)^+$. Comparison of ^1H and ^{13}C spectral data indicated that acid **3** was closely related to the diol **1** previously isolated from *T. reticulatus*. The differences in structure were deduced from the following data. The presence of the α,β-unsaturated acid in **3** is supported by the ^{13}C NMR signals at δ 172.0, 114.9, and 162.6, an IR stretch at 1640 cm^{-1}, and ^1H NMR signals at δ 5.74 (br s, 1H) and 2.22 (s, 3H). Treatment of **3** with diazomethane gave the expected methyl ester. Resolution enhancement of two broad signals at δ 4.99 (br s, 1H) and 5.33 (br s, 1H) in the ^1H NMR spectrum revealed a mutually coupled doublet and triplet, respectively ($J_{6,7} = 1.5$ Hz), similar to the signals assigned to H-6 and H-7 in diol **1.** The downfield shift of H-7 (δ 4.99 in **3** compared to 3.67 in diol **1** along with the appearance of a methyl singlet at δ 2.05 suggested esterification of the alcohol at C-7 by acetate. However, a long-range 2-D carbon–hydrogen correlation experiment revealed that the isovalerate ester was attached at C-7 and the acetate ester was at C-6, which was not the expected substitution pattern. This experiment showed that the ester carbonyl signal at δ 169.6 was coupled to both the acetate methyl signal at δ 2.05 and the methine signal at 5.33 (t, 1H, $J = 1.5$ Hz, H-6), while the ester carbonyl signal at δ 171.8 was coupled to the isovalerate α-methylene signal at 2.25 and the methine signal at 4.99 (d, 1H, $J = 1.5$ Hz, H-7). All other data are consistent with the proposed structure for 6β-acetoxy-7α-isovaleroxylabda-8,13-dien-15-oic acid (**3**).

The minor component of the organic extract was the sterol tetraacetate **4,** which was isolated as an oil, $[\alpha]_D = +29.7$. The molecular formula, $C_{35}H_{54}O_9$, was established by EI-MS [$m/z = 601.3740$, $(M\text{-}OH)^+$]. The ^1H NMR spectrum displayed signals at δ 0.86 (d, 3H, H-26), 0.87 (d, 3H, H-27), 0.92 (d, 3H, H-21), and 1.09 (s, 3H), representing four of the five methyl groups expected

for a cholestane sterol, the missing signal being that of the C-18 methyl group that is normally at δ 0.6 (s, 3H). Four methyl signals at δ 2.03 (3H), 2.04 (6H), and 2.10 (3H) indicated that four acetate groups were present. The presence of mutually coupled signals at δ 4.27 (d, 1H, J = 12.4 Hz) and 4.38 (d, 1H, J = 12.4 Hz) suggested that the C-18 methyl group was replaced by a $-CH_2OAc$ group. A signal at δ 4.68 (dd, 1H, J = 11.2, 5.1 Hz) was coupled to signals at 1.90 (m, 1H, H-11α) and 1.56 (m, 1H, H-11β), and the latter signal was in turn coupled to a signal at 1.40 (m, 1H, H-9), indicating the presence of an equatorial acetate group at C-12. The H-7 signal at δ 5.14 (br dd, 1H, J = 8.7, 2 Hz), was coupled to both the H-8 (J = 8.7 Hz) and the H-6 (J = 2 Hz) signals and also, through homoallylic coupling, to a proton signal at 2.05 (H-4α). The olefinic proton signal at δ 5.39 (br s, 1H, H-6) also showed allylic coupling to H-4α. The small coupling constant between H-6 and H-7 is consistent with an allylic, axial acetoxy group as described by Fattorusso *et al.* (1985). The signal at δ 3.27 (tt, 1H, J = 12, 4 Hz) is characteristic of the H-3 proton in a 3β-hydroxy sterol. The proton signal at δ 3.27 was coupled to a signal at 2.15 (H-2), which was in turn coupled to the remaining downfield signal at 5.21 (br d, 1H, J = 11 Hz, H-1), which required a 1β-acetoxy group. The remaining data were consistent with the structure of 1β,7β,12β,18-tetraacetoxycholest-5-en-3β-ol (**4**).

Localization of Metabolites. Diol **1**, the major secondary metabolite of *T. reticulatus*, was found only in the extracts of the foot and mantle, while the viscera contained none of the compound. The mucus produced by *T. reticulatus* when placed in a bag during collection also contained diol **1**, but the amount of mucus produced in this manner is less than would have been obtained by deliberately molesting the animals. Diterpene **3** and sterol **4** were found only in extracts of the foot and mantle of *T. conica*, but no mucus was collected from *T. conica*. The distribution of these metabolites is shown in Table 1.

TABLE 1. DISTRIBUTION OF DIOL **1** IN TISSUES (N = 7) AND MUCUS (N = 115) OF *T. reticulatus* AND OF ACID **3** AND STEROL **4** IN TISSUES OF *T. conica* (N = 33)

	Diol **1**	Acid **3**	Sterol **4**
Foot	260 μg/animal	100 μg/animal	+[a]
Mantle	4.0 mg/animal	400 μg/animal	+
Viscera	0	0	–
Shell	0	0	–
Mucus	78 μg/animal		

[a]Visible by thin-layer chromatography; –, not visible by thin-layer chromatography.

Seastar Response to T. reticulatus. When the seastar *Astrometis* sp. encountered the limpet *T. reticulatus*, the seastar retracted its arm by curling it up and backwards to avoid further contact. The seastar then changed direction to avoid further contact. When *Collisella limulata* or *C. scabra* were encountered, the seastar did not retract its arm but proceeded to crawl over the limpets. The cotton swab containing the mucus of *T. reticulatus* caused a similar arm-curling avoidance response to that caused by the whole animal. The cotton swabs containing mucus from *C. limulata* were not avoided and were crawled over.

Fish Feeding Deterrence. All krill pellets treated with diterpenoids **1** and **2** from *T. reticulatus* were readily eaten by the wrasse, *Thallasoma lunare*; these compounds did not appear to inhibit fish feeding. However, when the krill pellets

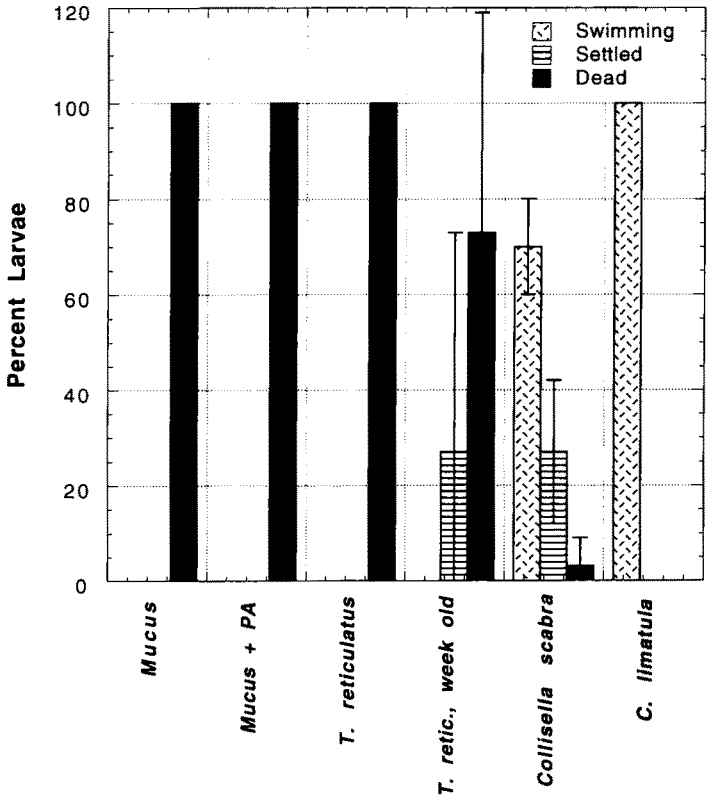

FIG. 2. The effect of *Colisella limatula, C. scabra, Trimusculus reticulatus*, mucus of *T. reticulatus*, mucus + palmitoleic acid (PA), and "aged" (1 week) *T. reticulatus* on *Phragmatopoma californica* larvae ($N = 30$).

were treated with limatulone (**5**), a known feeding inhibitor, the pellets were tasted and rejected by the wrasse.

Inhibition of Larval Settlement. The presence of a live individual of *T. reticulatus* was toxic to *Phragmatopoma californica* larvae. The presence of dried mucus collected from one individual limpet also caused 100% larval mortality. If the pulmonate limpet was kept in captivity for one week before carrying out the experiment, some of the effect was lost. The presence of the sympatric limpets *Collisella scabra* and *C. limatula* had no effect on the larvae. These results are summarized in Figure 2. The purified metabolite **1** from *T. reticulatus* had the same effect on the larvae as did the presence of the limpet or its mucus. At concentrations of 100 μg/treatment or greater, diol **1** caused 100% mortality (Figure 3). The effect was observed to drop off at 50 μg/treatment. However, when palmitoleic acid was added to the treatment and the larvae were induced to settle, 50% settled abnormally (Figure 4). Ether (100 μl) was used as a negative control, and when applied, 100% of the larvae remained swimming.

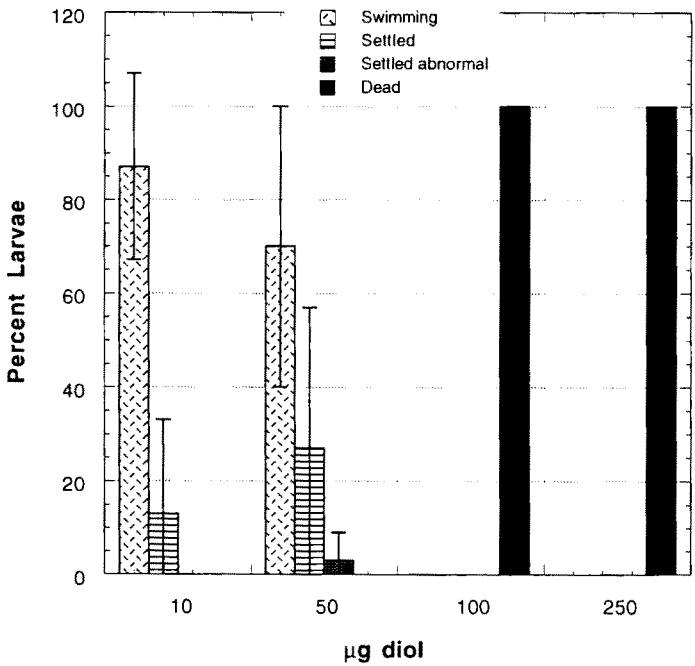

FIG. 3. The effect of diol **1** (micrograms per gram of sand) on *Phragmatopoma californica* larvae (N = 30).

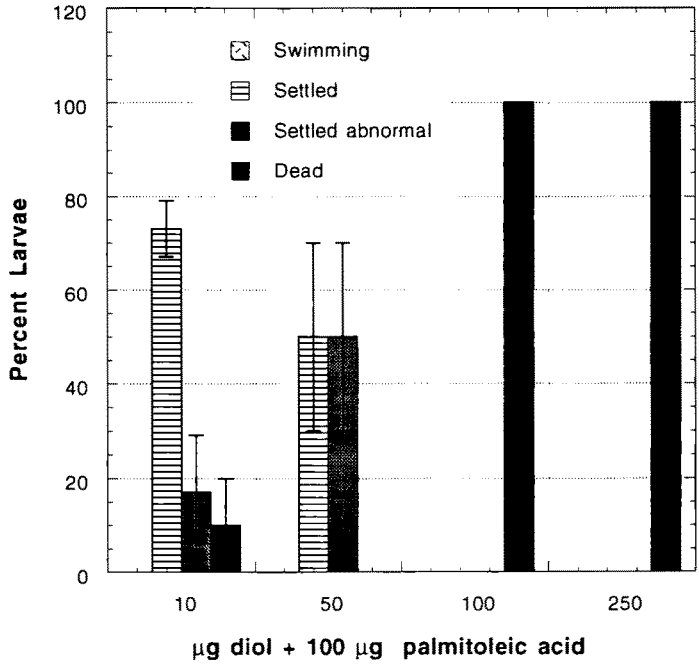

FIG. 4. The effect of diol **1** (micrograms per gram of sand) and palmitoleic acid (100 µg/g of sand) on *Phragmatopoma californica* larvae ($N = 30$).

When 100 µg of palmitoleic acid was used alone, 100% of the larvae settled normally. Acid **3** from *T. conica* did not cause larval mortality. However, it did appear to have some effect on settling, and at 100 µg/treatment, acid **3** caused 50% of the larvae to settle abnormally.

DISCUSSION

A careful investigation of the localization of the secondary metabolites of *T. reticulatus* revealed that the diterpenes were heavily concentrated in the mantle, foot, and mucus of the limpet. Neither the viscera nor the shell contained any of the chemicals. Another species of trimusculid, *T. conica* from New Zealand, was also collected to determine whether it contained similar compounds. The pulmonate limpets from New Zealand contained only one diterpene, the acid **3**, which was very closely related to those found in the California species, together with the sterol **4**. Similarly, the diterpene **3** and **4** were found

to be present only in extracts of the foot, mantle, and mucus. The localization of the diterpenes in the most exposed areas of the limpet suggests that they are deployed externally, probably in the mucus.

The compounds were screened in a number of bioassays to test their effectiveness as either defensive chemicals or antifouling agents. We observed the mucus of *T. reticulatus* to be repugnant to the sympatric seastar *Astrometis* sp., (cf. Rice, 1985), but diol **1** alone did not elicit the same response in the echinoderm. Insufficient quantities of diacetate **2** prevented similar tests. This may be due to the lack of an adequate carrier to mimic a mucus for presentation of diol **1** to the seastar. It is also feasible that more than one component is necessary in the mucus to repel seastars. The secondary metabolites did not inhibit feeding in the tropical rainbow wrasse, *Thallasoma lunare*. However, diterpene **1** was found to be highly toxic to veliger larvae of the sabellarid tube worm *Phragmatopoma californica*, which forms large reefs in the intertidal zone in proximity to colonies of *T. reticulatus*. The limpet, its mucus, or diol **1** at natural concentrations was also extremely larvicidal, while the prosobranch limpets caused no effect. In those experiments in which a settling factor, palmitoleic acid, was added and the *P. californica* larvae were induced to settle onto sand containing the diterpene, abnormal settlement was observed. This manifested itself in a failure to metamorphose, which included an inability to shed provisional setae or not rotating the larval tentacles anteriorly. Thus, a mucus secretion containing diterpene **1** would be very effective in preventing settling of a potential competitor. The lack of toxicity observed when using an individual *T. reticulatus* that had been starved for one week suggests that the concentration of secondary metabolites may have decreased to below the threshold required for efficacy and that dietary intake may be required for the production of diol **1.**

A second species, *T. conica*, which occurs in New Zealand, was found to contain diterpene **3,** which was closely related to the diterpenes found in *T. reticulatus*. Dissection and analysis showed similar localization of metabolites in the foot, mantle, and mucus. However, acid **3** was not effective against veliger larvae of the California tube worm. This lack of activity might be expected since *T. conica* has coevolved with different intertidal competitors in New Zealand.

CONCLUSION

The sessile nature of *T. reticulatus* and the fact that it filter feeds would make it particularly important for the limpet to keep the area around it free from fouling organisms. *P. californica* occurs in the same habitat and tidal range as *T. reticulatus*. The results show that natural concentrations of mucus or the presence of a live trimusculid limpet kills the larvae of *P. californica*. However,

in the presence of *Collisella scabra* or *C. limatula*, larvae of *P. californica* were not adversely affected. Clearly, the mucus of *T. reticulatus* is larvicidal to *P. californica*. The same activity is elicited by diol **1** purified from the mucus or whole animal, and it seems most likely that this compound is the larvicidal principle in the mucus. This may be an important strategy to the sessile, filter-feeding members of the genus *Trimusculus* that must keep their shells and the area around them free from other intertidal invertebrates in order to survive.

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ANT REPELLENT EFFECT OF THE STERNAL GLAND SECRETION OF *Polistes dominulus* (Christ) AND *P. sulcifer* (Zimmermann). (Hymenoptera: Vespidae)

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Abstract—The long-chain carboxylic acids identified in the sternal gland secretion of *Polistes dominulus* and *P. sulcifer* females were tested individually on three species of ants, *Crematogaster scutellaris*, *Formica cunicularia*, and *Lasius* sp., in order to verify if they have a repellent effect. The unsaturated acids (palmitoleic, linoleic, and oleic) act as repellents of all three ant species, while the saturated acids (lauric, myristic, palmitic, and stearic) have no effect. The mixture reproducing the secretion of the sternal glands of *P. dominulus* maintained its repellency for at least four days.

Key Words—Vespidae, Polistinae, *Polistes*, sternal glands, nest defense, ant repellents, allomones, unsaturated carboxylic acids.

INTRODUCTION

Several studies have shown that ants, especially in tropical and subtropical areas, exert high predatory pressure on the nests of social wasps, preying on their immature brood (Jeanne, 1975, 1979; Yamane, 1996). As active defense is less effective when only a few wasps constitute the colony, chemical defense against

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ants is used by many species. In at least some species of Stenogastrinae, the Dufour's gland secretion, consisting of a gelatinous substance, is used as a storage substrate for the larval food as well as a barrier against ants (Turillazzi, 1991, 1994). In females of the independently nest-founding Polistinae, the gland cells of the last gastral sternites secrete ant repellent substances (see Downing, 1991; Jeanne, 1996). By rubbing the abdomen on it, females apply these substances to the pedicel, which suspends the nest from the substrate and constitutes the only access to the nest for pedestrian predators. Recently, the production of ant-repellent secretions by gastral sternal glands has also been reported for two species belonging to the subfamily Vespinae (Martin, 1992).

Bioassays on several species of ants have been carried out using nest pedicels (Jeanne, 1970; Hermann and Dirks, 1974; Post and Jeanne, 1981; Turillazzi and Ugolini, 1979; Martin, 1992), glass capillaries or small wooden sticks stroked along the last abdominal sternites (Jeanne, 1970; Turillazzi and Ugolini, 1979; Post and Jeanne, 1981; Kojima, 1983; Martin, 1992), and solvent-extracted sternal glands (Keeping, 1990; Kojima, 1992). All the bioassays have shown that the sternal gland secretion and the substances coating the pedicel have a repellent effect on ants. Turillazzi and Ugolini (1979) and Kojima (1983) report that in *Polistes* and *Ropalidia*, respectively, the secretion loses activity a short time after the application, while Martin (1992) found that the repellent action lasts for several hours in *Vespa*.

From the sixth sternite glands of *Polistes fuscatus*, Post et al. (1984) isolated two components active against ants. One of these was identified as methyl hexadecanoate (methyl palmitate). Bioassays using methyl palmitate and some related methyl esters (methyl myristate, methyl linoleate, methyl linolenate, and methyl stearate) on several ants species (Post et al., 1984; Henderson and Jeanne, 1989) and on flies (Henderson et al., 1991) showed that only methyl myristate and methyl palmitate had a repellent effect, the former being more active than the latter. Also in these trials, the repellent effect lasted only for some hours.

More recently, hexadecanoic (palmitic) and octadecenoic (oleic) acid have been identified in the sixth sternite glands of *P. annularis* workers (Espelie and Hermann, 1990). The same compounds, together with other carboxylic acids (dodecanoic or lauric, tetradecanoic or myristic, hexadecenoic or palmitoleic, octadecadienoic or linoleic, and octadecanoic or stearic acid), have been found by Dani et al. (1995) in the fifth and sixth sternite glands of *P. dominulus*. *P. sulcifer*, a social parasite of *P. dominulus* (see Turillazzi, 1992; Cervo and Dani, 1996) has been shown to have most of the same fatty acids (palmitoleic, palmitic, oleic, and stearic) in its sternal glands also (Dani et al., 1995).

In the present research, bioassays have been performed in order to test if the acids found in the *P. dominulus* and *P. sulcifer* gastral sternite glands have an effect in repelling ants.

METHODS AND MATERIALS

Ant Species

The experiments were conducted in the laboratory at the Dipartimento di Biologia Animale e Genetica of Florence University. Most of the bioassays were carried out on *Crematogaster scutellaris*, but tests were also done on *Formica cunicularia* and a *Lasius* species. Turillazzi and Ugolini (1979) observed *Lasius* sp. and *Crematogaster* sp. invading nests of European *Polistes*, while Dani and Cervo (personal observation) have found nests invaded by *Formica* sp. *Crematogaster scutellaris* foragers were captured with an aspirator in the garden of the department and quickly used for the experiments. *Formica cunicularia* and *Lasius* sp. workers were collected in the Appennines near Pesaro (together with some immature brood coming from their nests) and used for the experiments the day after collection.

Experimental Apparatus

In all the experiments we used an experimental apparatus of the type employed by Turillazzi and Ugolini (1979). In each trial, about 40 ants were placed on an inverted Petri dish (9.5 cm in diameter), placed in the center of a bigger Petri dish (15 cm in diameter) containing water. A bridge was constructed with glass rods to permit the ants to escape from their water-surrounded dish. It consisted of a large glass rod (30 mm long and 2 mm in diameter), connected via a Y junction to two narrow glass rods (80 mm long and 1 mm in diameter). The end of the larger rod was put on the smaller Petri dish while the other two rods were placed on the border of the bigger Petri dish. Ants arriving at the Y junction had to make a decision to continue on one or the other branch to escape confinement. In each trial, before placing the bridge, one of the two smaller rods (experimental branch), near its junction with the main stem, was treated with a hexane solution containing synthetic carboxylic acids (the solvent was allowed to evaporate) while the other tube (control branch) was treated only with the same quantity of hexane. If the solution had a repellent effect, at the branching point, the ants tended to follow the control branch. Each trial lasted 20 min, unless all the ants crossed the branches in a shorter time. During the trial, the position of the experimental and control rods was alternated about every 5 min. If ants were walking on the rods, we waited until they had reached the end of the rods before alternating them. Four trials were made for each kind of experiment.

The results (i.e., the number of ants crossing the experimental and the control rods) of the four trials of each experiment were tested with the χ^2 homogeneity test. When the probability given by the test was not significant,

the data were cumulated and compared, using the G test, with a two-class uniform distribution (50%–50%), to find if a significantly different number of ants chose one of the two routes. When the probability given by the homogeneity test was significant, the G test was done for each single trial.

For graphic representation, we report the index of repellency of each acid as the percentage of ants that crossed the control route with respect to the total number of ants crossing both routes.

Experiments

Control Experiments. Prior to the experiments, nine control trials in which neither branch was treated were carried out on *Crematogaster scutellaris* to verify that the ants walked indiscriminantly on the two branches of the experimental device.

Effect of Each Acid. Oleic acid is the main component found in the sternal gland secretion of *P. dominulus*; the sixth sternite glands of one worker contained about 2.5 μg of this compound (Dani et al., 1995). In experiments on *Crematogaster scutellaris*, we tested separately lauric (dodecanoic), myristic (tetradecanoic), palmitoleic [(Z)-9-hexadecenoic], palmitic (hexadecanoic), linoleic [(Z,Z)-9,12-octadecadienoic], oleic [(Z)-9-octadecenoic], and stearic (octadecanoic) acid, by applying to the experimental branch 2.5, 25, or 250 μg of one of these acids diluted in 2μl of hexane (from solutions previously prepared), except that stearic and palmitic acid at 250 μg were each dissolved in 4 μl of hexane because of the limited solubility of these acids in hexane. Only the 250-μg quantity was tested on *Formica cunicularia* and on *Lasius* sp.

Duration of Repellent Effect. The sixth sternal glands of one *P. dominulus* worker contained, on average, the acids in the following percentages (Dani et al., 1995): lauric acid, 2.9% (195 ng); myristic acid, 5.9% (393 ng); palmitoleic acid, 4.1% (271 ng); palmitic acid, 21.4% (1410 ng) linoleic acid, 21.3% (1405 ng); oleic acid, 38.3% (2523 ng); and stearic acid, 5.9% (390 ng). A solution of pure acids, mixed in these proportions was tested on *Crematogaster scutellaris* by applying 2 μl of hexane containing the equivalent of the sixth sternite glands of 10 workers. The effect of this solution was tested at 0, 24, 48, and 96 hr after application to the rods.

RESULTS

Control Experiments. The control experiments showed that the device was balanced. In fact, although it was not possible to cumulate the data of the nine trials (homogeneity test; $\chi^2 = 6.58$, $P < 0.05$), in none of them did we find that significantly more ants preferred one of the two branches.

Effect of Each Acid. We report the average percentage (of the four trials) of *Crematogaster scutellaris* foragers that chose the control branch at the Y junction, using 2.5, 25, or 250 μg of each acid in Figure 1.

At 2.5 μg, palmitoleic, linoleic, and oleic acids had a highly repellent effect. It was not possible to cumulate the results of the four trials for palmitoleic acid (homogeneity test; $\chi^2 = 8.76$, $P < 0.01$), but in all of them significantly more than 50% of the ants crossed the control branch (Trial 1, $G = 21.26$, $P < 0.001$; Trial 2, $G = 33.00$, $P < 0.001$; Trial 3, $G = 39.57$, $P < 0.001$; Trial 4, $G = 6.70$, $P < 0.01$). The data of the other two unsaturated acids were cumulated ($\chi^2 = 3.12$, NS for linoleic acid; $\chi^2 = 1.65$, NS for oleic acid); in both the experiments significantly more ants followed the control branch ($G = 20.90$, $P < 0.001$ for linoleic acid; $G = 18.60$, $P < 0.001$ for oleic acid). No repellent effect was found for lauric, myristic, palmitic, and stearic acids.

At the 25-μg quantity, no ants at all crossed the rods to which linoleic acid ($\chi^2 = 0.01$, NS; $G = 250.93$, $P < 0.001$) or oleic acid ($\chi^2 = 0.21$, NS; $G = 141.42$, $P < 0.001$) had been applied, and only very few crossed the rods treated with palmitoleic acid ($\chi^2 = 0.31$, NS; $G = 215.19$, $P < 0.001$). No repellent effect was shown by lauric, myristic, palmitic, and stearic acids.

Because at 25 μg linoleic and oleic acids repelled all the ants (100%), we did not test these substances in the 250-μg experiments. In the trials with 250 μg, only very few ants chose the path with palmitoleic acid ($\chi^2 = 0.09$, NS; $G = 200.30$, $P < 0.001$), and no repellent effect was found for lauric, myristic, palmitic, and stearic acids.

Similar results were obtained in the experiments with *Formica cunicularia* (Figure 2) and with *Lasius* sp. (Figure 3) using 250 μg of acid. No *Formica cunicularia* ants used the branch treated with palmitoleic acid ($\chi^2 = 0.042$, NS; $G = 94.17$, $P < 0.001$). Only very few used that treated with oleic ($\chi^2 = 0.25$, NS; $G = 148.47$, $P < 0.001$) or linoleic acids ($\chi^2 = 0.12$, NS; $G = 158.20$, $P < 0.001$), and no repellent effect was shown by lauric, myristic, palmitic, and stearic acids.

Only a few *Lasius* sp. crossed the path treated with palmitoleic, linoleic, and oleic acids (respectively: $\chi^2 = 0.12$, NS; $G = 136.55$, $P < 0.001$; $\chi^2 = 0.08$, NS; $G = 148.02$, $P < 0.001$; $\chi^2 = 0.72$, NS; $G = 121.37$, $P < 0.001$), while lauric, myristic, palmitic, and stearic acids had no repellent effect.

In the case of *Crematogaster scutellaris*, we compared the results obtained in the experiments with the three different amounts of each acid. No differences in the repellent effect of the saturated acids were found between the 2.5- and 25-μg and between the 25-μg and 250-μg experiments. For linoleic and oleic acids, it was found that 25 μg of each substance had a greater repellent effect than 2.5 μg (respectively: $G = 87.92$, $P < 0.001$; $G = 65.11$, $P < 0.001$).

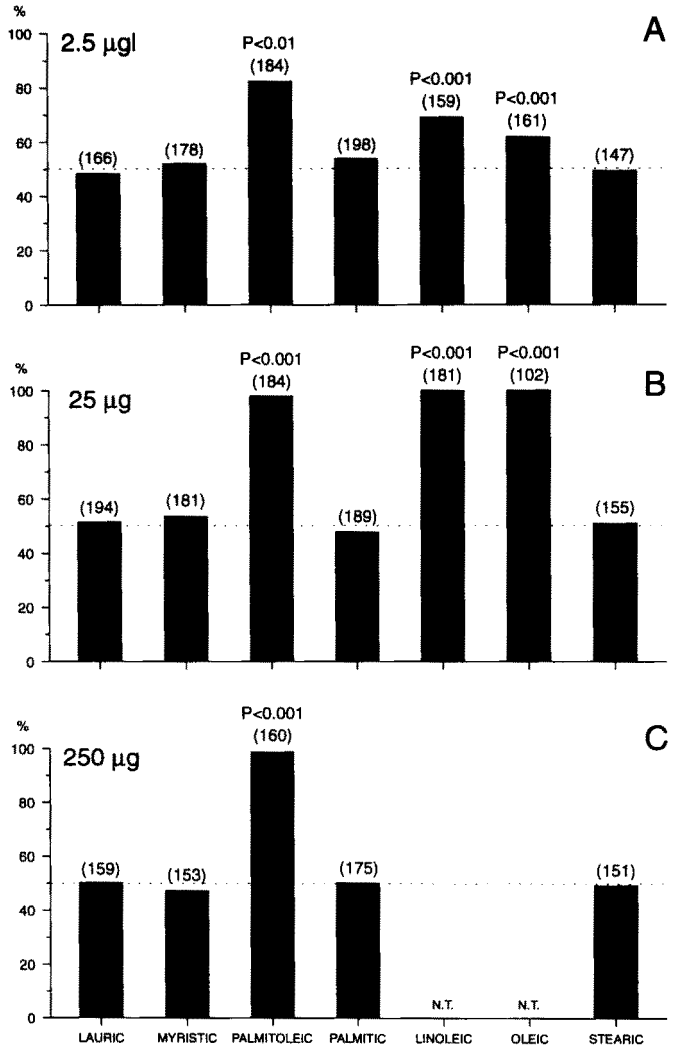

FIG. 1. Histograms showing the average percentage of *Crematogaster scutellaris* workers crossing the control branch with respect to the total number of ants crossing both control and experimental branches in the four trials using 2.5 µg (A), 25 µg (B), or 250 µg (C) of each acid. The total number of ants that crossed the experimental and the control branches in the four trials is given in parentheses. When the number of ants crossing the two branches significantly differs from a two-class uniform distribution (50%–50%) the result of the *G* test is given over that bar. Since in the 25-µg experiments linoleic and oleic acids repelled all the ants, these acids were not tested in the 250-µg experiments (N.T.).

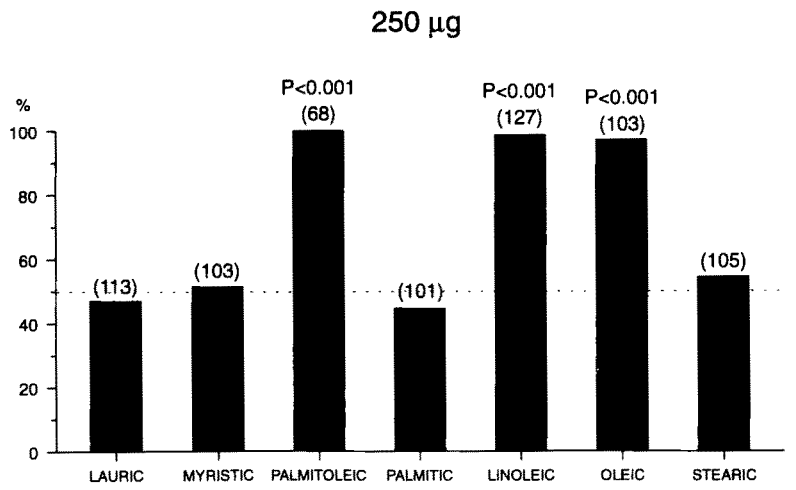

FIG. 2. Histogram showing the average percentage of *Formica cunicularia* workers crossing the control branch with respect to the total number of ants crossing both control and experimental branches in the four trials done using 250 μg of each acid. The total number of ants that crossed the experimental and the control branches in the four trials is given in parentheses. When the number of ants crossing the two branches significantly differs from a two-class uniform distribution (50%–50%) the result of the G test is given over that bar.

In three of the four trials with 2.5 μg (the result cannot be cumulated, see above), palmitoleic acid had less repellent effect than in the 25-μg experiment (Trial 1, $G = 13.13$, $P < 0.001$; Trial 2, $G = 38.63$, $P < 0.001$; Trial 3, $G = 4.06$, $P < 0.05$), while for the fourth trial no differences were found.

We conclude that the unsaturated acids (palmitoleic, linoleic, and oleic) have a strongly repellent effect on ants of the three species, while the saturated acids (lauric, myristic, palmitic, and stearic) have no effect, even if applied in a large amount. Ants reaching the surface covered with the unsaturated acids usually retreated and spent considerable time cleaning their antennae. Despite the fact that the applied surface seemed to be tacky and ants seemed to have difficulty walking on it, they did not become trapped by it.

The results obtained for each acid in the 2.5-μg experiments on *Crematogaster scutellaris* were compared. No statistical differences were found between the effects of the saturated acids. Comparing the effect of the unsaturated acids, we found no statistical differences between oleic and linoleic acid, while in three trials, palmitoleic acid was more repellent than oleic and linoleic ($G = 4.04$, $P < 0.05$; $G = 10.41$, $P < 0.01$; $G = 15.28$, $P < 0.001$ for the comparison between linoleic and palmitoleic acid; and $G = 5.32$, $P < 0.05$; $G = 12.35$,

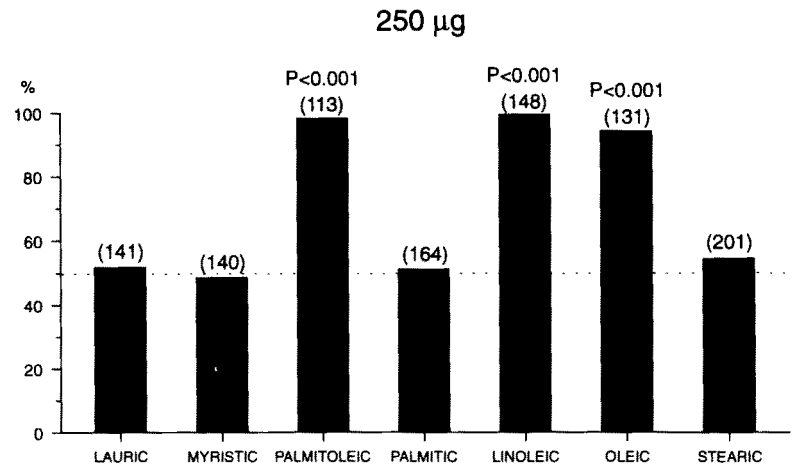

FIG. 3. Histogram showing the average percentage of the *Lasius* sp. workers crossing the control branch with respect to the total number of ants crossing both control and experimental branches in the four trials done using 250 µg of each acids. The total number of ants that crossed the experimental and the control branches in the four trials is given in parentheses. When the number of ants crossing the two branches significantly differs from a two-class uniform distribution (50%—50%) the result of the G test is given over that bar.

$P < 0.001$; $G = 17.47$, $P < 0.001$ for the comparison between oleic and palmitoleic acid). We found a statistical difference every time we compared the effect of a saturated acid with that of linoleic acid ($G = 12.31$, $P < 0.001$, for lauric acid; $G = 8.64$, $P < 0.01$, for myristic acid; $G = 6.18$, $P < 0.02$, for palmitic acid; $G = 10.60$, $P < 0.01$ for stearic acid), and similarly with oleic acid ($G = 10.33$, $P < 0.01$, for lauric acid; $G = 6.86$, $P < 0.01$, for myristic acid; $G = 4.58$, $P < 0.05$, for palmitic acid; and $G = 8.74$, $P < 0.01$, for stearic acid). Indeed, we found a statistical difference ($0.05 < P < 0.001$) any time we compared the results of the four trials with palmitoleic acid with those of the experiments with the saturated acid, with the exception of one comparison between a trial of palmitoleic and palmitic acid.

In the 25-µg experiments on *Cremastogaster scutellaris*, there were no statistical differences between the effects of the saturated acids; while for the unsaturated ones we found that linoleic acid was more repellent than palmitoleic ($G = 5.54$, $P < 0.02$). We found statistical differences in any comparison between saturated and unsaturated acids (G test, $P < 0.001$ for the 12 comparisons).

In the 250-µg experiments on *Cremastogaster scutellaris*, no differences

TABLE 1. DURATION OF REPELLENCY EFFECT ON *Crematogaster scutellaris* WORKERS OF A SOLUTION OF SYNTHETIC ACIDS REPRODUCING PROPORTIONS OF ACIDS IN STERNAL GLAND SECRETION OF 10 *Polistes dominulus* WORKERS[a]

	Time (hr) after application			
	0	24	48	96
Average percent repellency ± SD (Total number of ants crossing control and experimental branches)	78.5 ± 0.09 (107)	82 ± 0.08 (107)	86 ± 0.04 (133)	86.5 ± 0.08 (135)

[a]Each experiment consisted of four trials. The mean percentage (with the standard deviation) of ants crossing the control branch with respect to the total number crossing both experimental and control branches (in parantheses) is reported.

were found between the effects of the saturated acids. As oleic and linoleic acid were not tested at this amount (see above), comparisons between unsaturated acids were not done. Finally, palmitoleic acid was more repellent than any saturated acids (G test, $P < 0.001$ for the four comparisons).

The 250-μg experiments on *Formica cunicularia* and on *Lasius* sp. revealed no statistical differences between the saturated acids. For *F. cunicularia* we also found no differences between the unsaturated acids, while for *Lasius* we found that the linoleic acid is more active than oleic ($G = 0.63$, $P < 0.01$). For both species we always found a highly significant difference any time we compared the effect of an unsaturated with that of a saturated acid (G test, $P < 0.001$ for the 12 comparisons).

Duration of Repellent Effect. The repellency of the mixture (applied as a 10-worker equivalent) did not change much with time (Table 1). It initially increased a little (but not significantly) and then remained constant for at least four days after application.

DISCUSSION AND CONCLUSIONS

All the results indicate that the unsaturated carboxylic acids found in the fifth and sixth sternite glands of *P. dominulus* and *P. sulcifer* have an ant repellent effect, while the saturated acids are not repellent at all. The unsaturated carboxylic acids have a repellent effect even if tested in amounts similar to that contained in the sternal glands of just one female.

The saturated and unsaturated long-chain carboxylic acids differ in their physical and chemical properties. Physically, the saturated acids are solids and

the unsaturated acids are liquids at room temperature. Chemically, the unsaturated acids possess a reactive double bond and react with atmospheric oxygen. We do not think the physical state of the unsaturated acids is the cause of their repellency, and no oily surface is observable on the nest pedicel.

Myristoleic, palmitoleic, oleic, and linoleic acids appear in ants dead for several hours (Blum, 1970), and they induce necrophoric behavior in ants belonging to the genera *Pogonomyrmex*, *Solenopsis*, and *Myrmecia* (Wilson et al., 1958; Blum, 1970; Haskins and Haskins, 1974; Gordon, 1983). Oleic acid occurs in the eggs of the beetle *Gastrophysa cyanea*, where it acts as a feeding deterrent against several species of ants (Howard et al., 1982). Fatty acids from the cornicles of aphids have a repellent effect on *Monomorium* workers (Phelan, personal communication in Howard et al., 1982). Howard et al. (1982) suggested that the use of fatty acids as ant repellents is a widespread phenomenon among insects, and our results seem to support this hypothesis.

Turillazzi and Ugolini (1979) and Kojima (1983) found that the repellent effect of the sternal gland secretion is not long lasting, suggesting that the frequent gastral rubbing performed by females on the pedicel is related to the rapid loss of repellency of the secretion. Similar experiments by Post et al. (1984) and Henderson and Jeanne (1989) using the methyl esters of some of the acids we tested in this research showed repellent action for only methyl myristate and methyl palmitate, but it was very limited.

Besides the production of allomones, an involvement of the sternal glands of *Polistes* wasps in communication between nestmates has also been supposed (West-Eberhard, 1982; Downing and Jeanne, 1983). For the social parasite *P. sulcifer* it has been suggested that the sternal glands are involved in the chemical control of the host colony (Turillazzi et al., 1990). Further studies are necessary to verify if carboxylic acids may have some role in communication between nestmates.

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ISOLATION AND IDENTIFICATION OF HOUSE FLY, *Musca domestica* L., REPELLENTS FROM PEPPER TREE, *Schinus molle* L.

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Abstract—Foliage from the pepper tree, *Schinus molle* L., is traditionally used in Ethiopia to "repel" house flies, *Musca domestica* L. The volatile extracts of pepper tree leaves were shown to have repellent and feeding-deterrent activity against house flies in a two-choice laboratory bioassay. High-performance liquid chromatographic fractionation of steam-distilled volatiles from leaves, monitored by laboratory bioassays, demonstrated that bioactivity is associated with two compounds, *cis*-menth-2-en-1-ol and *trans*-piperitol. The absolute configuration of the latter was established as (1*S*,6*S*)-piperitol by comparison of acetyl lactate derivatives. Racemic compounds were synthesized from piperitone, and bioassays with house flies indicated *trans*-piperitol to be the most active house fly repellent.

Key Words—Pepper tree, *Schinus molle*, house fly, *Musca domestica*, *cis*-menth-2-en-1-ol, *trans*-piperitol, repellent, feeding deterrent.

INTRODUCTION

The pepper tree, *Schinus molle* L. (California or Australian pepper tree, Peruvian or American mastic tree) (Anacardiaceae), is native to the Peruvian Andes (Heywood, 1982) and is widely grown in tropical and subtropical countries (Zeven,

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1982). The tree grows 6–10 m high with a 60 to 90-cm-diameter trunk. The foliage consists of 12 to 20-cm-long, pinnate, feathery, green leaves. The flowers are yellow–white and develop into red fruits (drupes) (Graf, 1981).

Foliage of the pepper tree is reported to be a traditional source of repellents for house flies, *Musca domestica* L., in Ethiopia and some rural people drape branches and leaves over their heads to repel house flies. Pepper tree leaves are spread on eating tables, and occasionally in slaughter houses and meat-processing areas. A number of investigators have identified several chemical constituents of pepper tree volatiles (Aziz-Ur-Rahman et al., 1974; Bernhard et al., 1983; Delvalle et al., 1987; Dominguez et al., 1971; Hashim et al., 1980; Navarrete et al., 1989; Talenti et al., 1989), none of which has been reported as a repellent for house flies.

On the basis of Ethiopian tradition, our objective was to investigate the hypothesis that volatile chemical house fly repellents are present in the pepper tree. The hypothesis was strengthened by the results of preliminary field bioassays (conducted by K.N.S., J.H.B., and T.A.), which showed that chopped leaves and berries of pepper trees, and crude ethanolic extracts thereof, were repellent to house flies in Ethiopia. This paper reports the laboratory bioassay, isolation, and identification of candidate house fly repellents from pepper tree leaves (Wimalaratne, 1993).

METHODS AND MATERIALS

Preparation of Pepper Tree Extracts

Fresh pepper tree leaves were obtained from 1 to 2-year-old trees grown in a greenhouse at Simon Fraser University, British Columbia, Canada. Shoots with matured leaves were separated intact from the trees, and were stored in sealed polyethylene bags at −20°C. Pepper tree leaves (400 g) were blended with distilled water (1500 ml) until a pulp was formed. Leaf volatiles were extracted into pentane from the macerated pulp using a modified Likens-Nickerson simultaneous distillation–solvent extraction drive (Flath and Forrey, 1977) over a period of 6 hr. The pentane extract was dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated by distillation of the pentane through a Dufton column, and the residual solvents were removed by aspiration with nitrogen.

Bioassay

A two-choice laboratory bioassay was developed in which wooden cages (16 × 16 × 13 cm) were used as bioassay chambers. The back wall of each cage was a fine plastic screen and the front wall was a transparent Plexiglas

sheet with central hole (2 cm diam.), through which insects were released into the cage and which was then closed with a cork. The plastic sheet could also be raised, providing a means to place baits and water vials in the cage and to remove dead flies. A water vial with a cotton wick was kept inside the cage to maintain high relative humidity and to provide drinking water for the flies.

Two glass cover slips (2.2 × 2.2 cm) served as bait stations. Prior to bioassays the center of each cover slip was treated with a 50-μl drop of sucrose solution (sucrose–water 1 : 1 w/v); heating to dryness at 60–70°C in an oven left a sugar coating on the glass. Newly emerged adult house flies were obtained from the insectary at Simon Fraser University, British Columbia, Canada. For each bioassay replicate, 20 laboratory-reared, randomly selected flies (mixed sex and age) were released into a cage and held without food for 1 hr. One bait station was treated with the test stimulus (25 μl), and the other with solvent (25 μl). After evaporation of the solvent, the bait stations were placed 10 cm apart on the floor of the cage. The number of sustained fly contacts ≥ 10 sec on both bait stations were counted for 6 min. The proboscis was usually extended on such contacts. Each treatment was assayed with five groups of 20 flies each at room temperature and humidity. Bioactive constituents characteristically induced flies that made contact with the treated substrate to depart immediately (repellency), but if contact was sustained, extension of the proboscis and feeding was sometimes also repressed (deterrency) (Nordlund, 1981). Because the former response predominated, for convenience we refer to the combined effect as repellency.

Bioassay experiments were conducted to test the activity of steam-distilled volatile extracts, fractions, and two synthetic candidate repellents, *cis*-menth-2-en-1-ol **(I)** and *trans*-piperitol **(II)** (Figure 1). Known weights of racemic samples of **I** and **II** diluted in distilled hexane were assayed on house flies. Three series of experimental samples were prepared with **I** and **II** individually and a 1 : 1 w/w mixture of **I** and **II.** Dose–response experiments were conducted to determine the minimum quantity of each compound separately, and in combination, required to elicit significant repellency.

Percent responses (pooled data for the five groups of flies) to experimental stimuli were compared to those to crude extract stimuli by a z test ($\alpha = 0.05$) (Zar, 1984).

Isolation and Identification of Repellents

Gas–liquid chromatographic (GC) analysis was performed on a Hewlett-Packard 5880A gas chromatograph equipped with a DB-1 fused-silica capillary column. Matching retention times for isolated active components and authentic samples were performed on a Hewlett-Packard 5890A gas chromatograph using both DB-1 and DB-23 fused-silica capillary columns.

Fractionation of the steam-distilled volatile extract of pepper tree leaves was performed on a Waters LC625 high-performance liquid chromatograph (HPLC) at 1 ml/min with a Waters 486 variable wavelength UV–visible detector system set at 280 nm.

Electron impact ionization mass spectra (MS) were obtained on a Hewlett-Packard 5985B GC-mass spectrometer. NMR spectra of synthesized compounds were recorded with a Bruker 400 MHz instrument after D_2O exchange and chemical shifts were reported in parts per million (ppm). Infrared (IR) spectra were obtained on neat samples (NaCl plates) with a Perkin Elmer 1600 FTIR instrument.

Fractionation of Repellent Extracts

The crude extract of pepper tree leaves was fractionated by HPLC on a Nova pak silica column in a nonlinear step-gradient program to give seven fractions (5 ml each, ether–hexane: 1% at 5 min, 5% from 5 to 10 min, 10% from 10 to 15 min, 15% from 15 to 20 min, 20% from 20 to 25 min, 30% from 25 to 30 min, 100% from 30 to 35 min). Fractions to be subjected to GC analysis were concentrated by evaporation under a stream of nitrogen with ice cooling and were bioassayed on house flies. The behaviorally active fourth fraction obtained with 15% ether–hexane was refractionated by HPLC on a reverse-phase Nova pak C_{18} column (10 fractions, 1 ml each, water–acetonitrile: 15% isocratic for 10 min). Fractions were extracted into pentane, analyzed by GC, and bioassayed. The behaviorally active fourth fraction was refractionated by HPLC on a Nova pak CN-HP column (20 fractions, 0.5 ml each, λ-220 nm, 0.5 ml/min, ether–hexane: 1% at 2 min, 1% to 11% from 2 to 20 min). Fractions were analyzed by GC and bioassayed.

Microscale Analysis

Authentic samples of (1*R*,2*S*,5*R*)-(−)-menthol, (1*S*,2*R*,5*R*)-(+)-isomenthol, and (1*S*,2*R*,5*S*)-(+)-menthol were obtained from Aldrich Chem. Co., Milwaukee, Wisconsin.

Acetylation. The partially concentrated final active fraction was treated with an excess of acetic anhydride (0.3 ml), pyridine (0.3 ml), and 4-dimethylaminopyridine (~5 mg) and stirred overnight at room temperature. The reaction was quenched by adding water and the mixture extracted into hexane (4 × 0.5 ml). The hexane extract was washed successively with 10% HCl (5 × 0.5 ml), water (2 × 0.5 ml), saturated NaCl (0.5 ml), and dried over anhydrous Na_2SO_4, filtered, and concentrated. Product identity was confirmed by matching GC retention times with standards on DB-1 and DB-23 columns of different polarity and GC-MS fragmentation patterns.

Hydrogenation. Hydrogenation of the final active fraction was carried out in a thick-walled Reacti-vial (1 ml) equipped with a tight-fitting Teflon-lined septum. A mixture of the hexane–ether solution of the behaviorally active sample and 10% palladium on carbon (~2 mg) was pressurized with hydrogen and stirred for 1 hr. The product was filtered through glass wool and concentrated. Identification of the products was made by matching GC retention times with standards and GC-MS fragmentation patterns.

Hydrogenation of acetylated products of behaviorally active fractions and acetyl lactyl derivatives of synthetic *trans*-piperitol **(II)** [*trans*-3-methyl-6-(1-methylethyl)-2-cyclohexene-1-ol] were conducted according to the same procedure.

Oxidation. To the sample in dichloromethane (0.4 ml) a small amount of pyridinium chlorochromate (PCC, ~50 mg) was added and stirred for 2 hr at room temperature (Corey and Suggs, 1975). The mixture was diluted with anhydrous ether (0.25 ml), filtered through Florisil and concentrated.

Chiral Determination Using Acetyl S-Lactyl Chloride. Preparation of acetyl lactyl chloride (AclacCl) reagent and derivatization of compound **II** in the concentrated final behaviorally active fraction and synthetic standards were carried out as reported by Slessor et al. (1985). Separation of prepared acetyl lactyl derivatives of *trans*-piperitol was performed by HPLC using a Nova pak silica column (λ-220 nm, 5% ether/hexane isocratic).

Synthesis of cis- *and* trans-*Piperitol* ***(II)***

Piperitone (ICN, Inc., Costa Mesa, California) was reduced with $LiAlH_4$ to provide a mixture of *cis*- and *trans*-piperitol (Wheeler and Chung, 1969). The crude product was separated by column chromatography (Alumina, Fisher, 80–200 mesh) with pentane, ether, and methanol. The ratio of *cis* and *trans* unsaturated alcohols was 33 : 67, and the former eluted with pentane–ether (1 : 1), whereas the latter eluted with ether–methanol (6 : 4).

The first eluted *cis*-alcohol showed 99% purity by GC and exhibited the following spectra: IR (film) 3373, 2927, 2869, 2724, 1673, 1473, 1448, 1383, 1233, 1159, 1129, 1046, 1022, 956, 902, 847, 802 cm^{-1}; *m/e* (relative intensity); 154(M^+, 4), 139(28.5), 121(2.5), 112(9), 111(8), 97(5), 95(5), 94(3), 93(13), 92(2.5), 91(9), 85(7), 84(100), 83(35), 81(6), 79(9), 77(11), 71(9), 69(9), 67(5), 65(4), 56(10), 55(13.5), 53(6), 51(3), 43(13). ^1H NMR ($CDCl_3$) ppm 0.955 (3H, $-CH_3$, d, J = 6.5 Hz), 0.995 (3H, $-CH_3$, d, J = 6.5 Hz), 1.31 (1H, m), 1.6–1.8 (2H, m), 1.69 [3H, $-CH = C(C\underline{H}_3)-$, s] 1.9–2.1 (3H, m), 4.12 [1H, $-C(OH)\underline{H}-$, m], 5.63 (1H, $=C\underline{H}-$, m).

The *trans*-alcohol **(II)** was 99% pure by GC and exhibited the following spectra: IR (film) 3318, 2930, 2871, 2724, 2341, 1676, 1466, 1434, 1384, 1297, 1232, 1158, 1049, 1027, 985, 899, 840 cm^{-1}; *m/e* (relative intensity);

154(M^+, 8.6), 139(39), 136(11), 121(9.5), 112(10), 111(11.6), 97(7), 95(7), 94(6.5), 93(42), 92(10), 91(33), 85(6), 84(100), 83(43), 81(8), 79(16.5), 78(6), 77(34), 71(11.5), 69(12), 67(8), 65(10.6), 56(12), 55(17.4), 53(10), 51(7), 43(22.4). ^{1}H NMR ($CDCl_3$) ppm 0.84 (3H, $-C\underline{H}_3$, d, J = 7 Hz), 0.96 (3H, $-C\underline{H}_3$, d, J = 7 Hz), 1.24 (1H, m), 1.6–1.75 (2H, m), 1.67 [3H, $=C(C\underline{H}_3)-$, s) 1.9–2.05 (3H, m), 4.01 [1H, $-C(OH)\underline{H}-$, d, J = 7.5 Hz), 5.38 (1H, $=C\underline{H}-$, m).

Synthesis of cis-*Menth-2-en-1-ol* ***(I)***

The resulting *cis*-piperitol was converted to compound I by acid catalyzed rearrangement as reported for amitinol (Francke et al., 1980). A solution of 0.5% BF_3 in CH_2Cl_2 (150 ml) at −78°C was added, with stirring, to a solution of *cis*-piperitol (0.5 g, 0.003 mol) (obtained from the previous experiment) in CH_2Cl_2 (5 ml). The mixture was stirred for 2 hr at about −40°C. The reaction was monitored by GC every 15 min. The reaction was quenched with water (100 ml), and the organic layer was separated. The aqueous layer was extracted with CH_2Cl_2 (2 × 25 ml). The CH_2Cl_2 extract was washed with water (4 × 25 ml), saturated $NaHCO_3$ (25 ml), and saturated NaCl (25 ml), and dried over anhydrous Na_2SO_4. The solvent was partially removed on a rotary evaporator.

The mixture was treated with excess acetic anhydride (20 ml) and pyridine (15 ml) and was stirred overnight at room temperature. The reaction was quenched with water (50 ml) and the mixture extracted with hexane (4 × 25 ml). The hexane extract was washed with water (6 × 25 ml), and saturated NaCl and dried over anhydrous Na_2SO_4. The solution was filtered, evaporated and chromatographed on a silica gel column with hexane–ether (8:2), yielding 0.13 g of product with 99% purity by GC. This product exhibited the following spectra: IR (film) 3353, 2959, 2871, 1744, 1726, 1643, 1464, 1384, 1368, 1230, 1206, 1170, 1122, 1105, 1084, 1063, 1012, 996, 968, 944, 906, 863, 818, 807, 734 cm^{-1}; *m/e* (relative intensity); 154(M^+, 6.4), 139(39), 136(17.5), 121(30), 111(38), 107(8), 97(13), 96(12), 95(25), 94(45), 93(65), 92(17), 91(29), 86(6), 84(33), 83(33), 82(7), 81(44.5), 80(9.5), 79(64), 78(8), 77(37), 71(65), 70(7), 69(55), 68(8), 67(27), 66(6.5), 65(14), 58(12), 57(6), 55(31), 53(11.5), 51(7), 45(5), 43(100). ^{1}H NMR ($CDCl_3$) ppm 0.89 (3H, $-C\underline{H}_3$, d, J = 7 Hz), 0.91 (3H, $-C\underline{H}_3$, d, J = 7 Hz), 1.27 [3H, $-C(OH)C\underline{H}_3-$, s), 1.35–1.55 (2H, m), 1.65 (2H, m), 1.85 (2H, m), 5.65 (2H, m).

RESULTS AND DISCUSSION

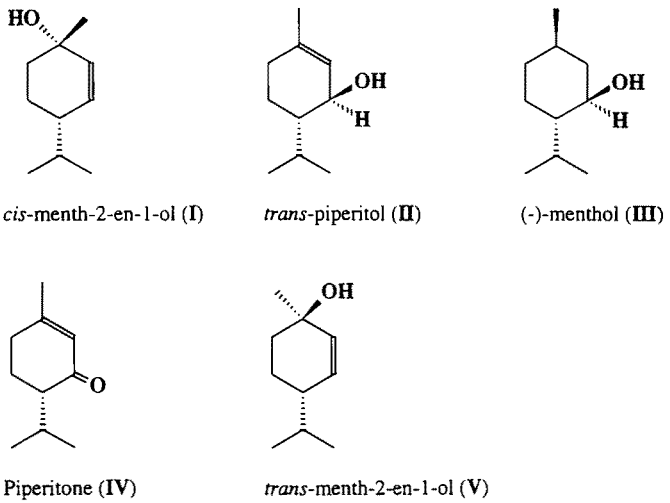
Maximal activity in 6-min bioassays was obtained with 0.3 mg of steam-distilled oils. Only fraction 14 of the final HPLC fractionation was behaviorally active on house flies and its GC trace showed two major compounds, **I** and **II**

(Figure 1) (3.81 and 4.91 min, respectively, DB-1 column, 30 m × 0.25 mm ID, 2 min at 80°C, 10°C/min to 210°C). Compounds **I** and **II** were present in 0.2% and 0.03% in the steam-distilled oils of pepper tree as determined by GC.

Compounds **I** and **II** had been observed in all fractions exhibiting repellent activity but in different ratios throughout the fractionations, suggestive of compound lability and interconversion.

Identification of I and II. The MS spectra of compounds **I** and **II** indicated the molecular weight of both compounds as 154, corresponding to a molecular composition of $C_{10}H_{18}O$ with two degrees of unsaturation. The fragmentation patterns in both spectra indicated the loss of a methyl (*m/e* 139) and water (*m/e* 121), indicating the presence of both methyl and hydroxyl functionalities. Analysis of the acetylated product of the behaviorally active fraction by GC indicated two major detector responses, one at a retention time identical to compound **I** and another with a retention time (6.70 min) later than compound **II.** The mass spectrum of the early retention compound was identical to that of compound **I,** indicating that compound **I** had not been acetylated and was suggestive of a tertiary alcohol, whereas the acetylation of compound **II** was suggestive of the presence of a primary or secondary alcohol.

Hydrogenation of the biologically active fraction showed three major GC peaks (3.98, 4.45, and 4.66 min), all of which were different from the **I** and **II.** Only the earliest-eluting peak was identical to that obtained from hydrogenation of the acetylated fraction, indicating that it was derived from the unace-



cis-menth-2-en-1-ol (I) *trans*-piperitol (II) (-)-menthol (III)

Piperitone (IV) *trans*-menth-2-en-1-ol (V)

FIG. 1. Structures of compounds **I** and **II** isolated from pepper trees, and related compounds.

tylated tertiary alcohol, **I.** The presence of only one hydrogenated product from **I** seemed to infer a disubstituted ring double bond, which would not lead to stereoisomers on reduction. The secondary alcohol, **II,** on the other hand, generated two stereoisomers, indicating the presence of a trisubstituted double bond. One of the products of the hydrogenation of **II** gave GC retention (4.45 min) and MS data identical to menthol (**III,** Figure 1), providing a ring size and substitution pattern for **II.** Oxidation of **I** and **II** produced a single product whose GC retention times (5.39 min) and MS fragmentation pattern was identical to piperitone (**IV,** Figure 1). The secondary alcohol **(II)** present in the behaviorally active fraction must therefore be *trans*-piperitol.

The hydroxyl group of piperitol migrates between the C-1 and C-3 positions in acidic media (Clark et al., 1977), suggesting either *cis* or *trans*-menth-2-en-1-ol as the structure of the tertiary alcohol **I.** Synthetic *cis*-menth-2-en-1-ol and *trans*-piperitol showed identical GC retention characteristics on two different column phases (DB-1 and DB-23) and gave identical MS fragmentation patterns to the two behaviorally active alcohols **I** and **II.**

Determination of Chirality of trans-Piperitol from Pepper Trees. The acetyl-lactated product of synthetic *trans*-piperitol (**VII, XII,** Figure 2) showed two signals in the gas chromatogram. Hydrogentation of HPLC separated diastereomers (**VIII, XIII,** Figure 2) each generated two compounds (**IX** and **X, XIV** and **XV,** Figure 2) with distinct GC retention times (DB-1 column, 30 m × 0.25 mm ID, 145°C isothermal, 5.10, 5.25, 5.19, and 5.31 min for **IX, X, XIV,** and **XV,** respectively) and appropriate MS fragmentation patterns. GC retention times and MS fragmentation of the products **IX, XIV,** and **XV** corresponded to those of the acetyl lactates of authentic samples of (1*R*,2*S*,5*R*)-(−)-menthol **(XVI),** (1*S*,2*R*,5*S*)-(+)-menthol **(XVII),** and (1*S*,2*R*,5*R*)-(+)-isomenthol **(XVIII),** respectively, indicating that compounds **XIII** and **VIII** were the acetyl lactates of (1*R*,6*R*)-piperitol and (1*S*,6*S*)-piperitol. Derivatization of the final behaviorally active fraction produced a single product that was identical in GC retention time and MS fragmentation to that of compound **VIII.** Hydrogenation of **VIII,** derived from the natural compound **II,** produced two products identical with those of compounds **IX** and **X,** respectively. These results established the structure of compound **II** to be (1*S*,6*S*)-piperitol.

Bioassay of Synthetic Compounds. Synthetic compounds *cis*-menth-2-en-1-ol **(I)** and *trans*-piperitol **(II)** had both repellent and feeding deterrent effects on house flies, eliciting maximum response at doses of 1 and 0.1 mg, respectively (Table 1). The latter repressed both sustained contacts and extension of the probosis for the full 6-min test period, whereas the former induced such a maximal response for only 4 min at the 0.1-mg dose. Thus, *trans*-piperitol appears to be the major repellent in steam-distilled volatile extracts of pepper tree leaves.

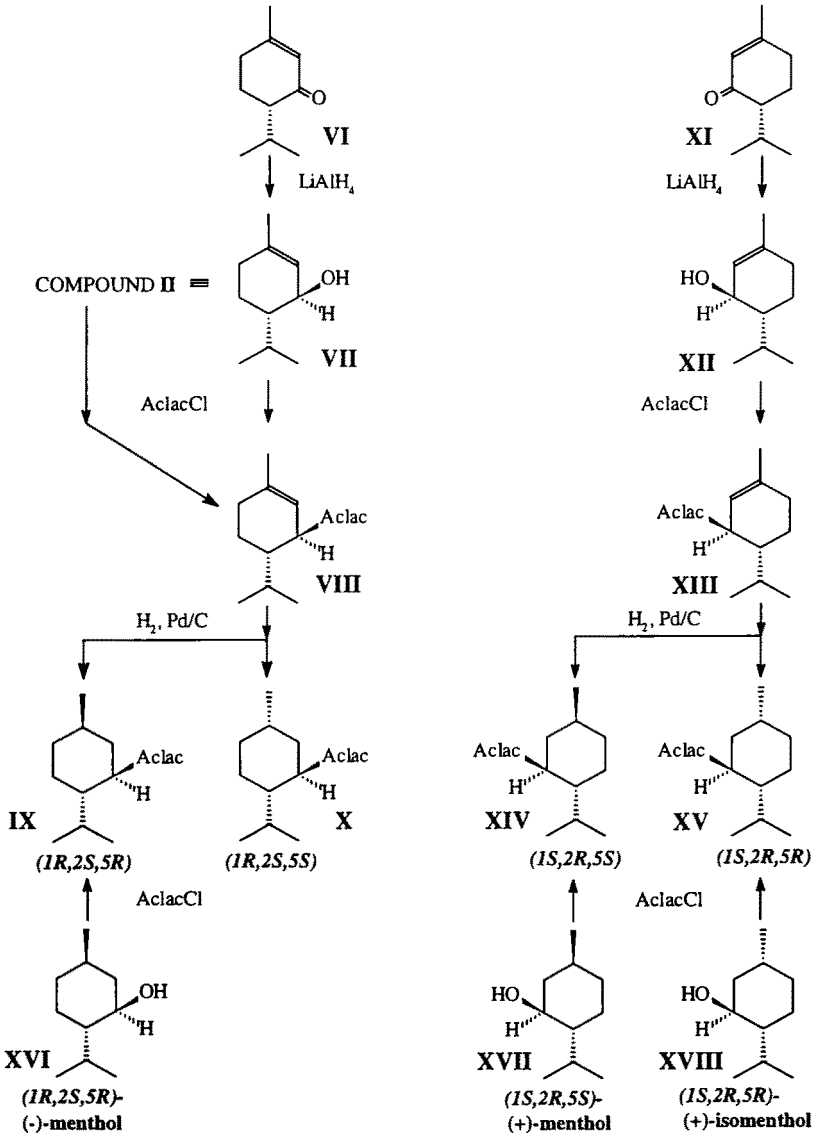


FIG. 2. Determination of chirality of compound **II**.

TABLE 1. RESPONSE OF HOUSE FLIES TO SUGAR-COATED SOLVENT CONTROL STATIONS IN TWO-CHOICE LABORATORY BIOASSAYS[a]

Dose (mg/25 μl)	Percent of total sustained contacts on solvent control station[b]			
	Crude pepper tree oil	Compound **I**	Compound **II**	1 : 1 ratio mixture of **I** and **II**
0.001	—	62***	60***	60***
0.01	—	78**	66***	83**
0.05	—	—	—	100
0.1	—	93	100	100
0.8	100	—	—	—
1	—	100	100	—

[a]One of the sugar-coated experimental stations was treated with various doses of crude pepper tree oil or synthetic compounds **I** (*cis*-menth-2-en-1-ol), **II** (*trans*-piperitol) and 1 : 1 ratio mixture of **I** and **II**.

[b]Significant differences (*z* test) from those to the crude pepper tree oil indicated by $^{**}P < 0.01$ or $^{***}P < 0.001$.

A higher response was observed for the binary mixture of the alcohols than for the individual compounds, eliciting maximum response to 0.05-mg doses, and complete repellency for 4 min to 0.01-mg doses. Volatility and evaporation of the pure alcohols is the likely cause of the decline in activity observed at low concentrations in comparison to extracts that contain many other components acting in a "keeper" role.

Practical Implications. The bioactivity of **I** and **II** suggests that they could be the basis for the folkloric use of cut pepper tree leaves and branches to repel house flies in Ethiopia. However, their volatility and hence short-lived activity in pure form indicates that they would need to be prepared in a very well-controlled, slow-release formulations to have any practical utility. It has recently been found that a powder made from green pepper tree berries can protect bean seeds, *Phaseolus vulgaris* L., for at least three months from infestation by bruchid beetles (T. Abate, unpublished results). This finding suggests that **I** and **II** released at slow rates from the green seed powder could have repellent properties for insects other than house flies. Alternatively, there may be additional volatile or nonvolatile components in pepper tree berries that have potential practical use as insect repellents.

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EFFECTS OF OCTADECANOID METABOLITES AND INHIBITORS ON INDUCED NICOTINE ACCUMULATION IN *Nicotiana sylvestris*

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Abstract—We examined the effects of inhibitors of the octadecanoid pathway (*n*-propyl gallate, acetosalicylic acid, salicylhydroxamic acid, methyl salicylate, and antipyrine) on wound- and jasmonate-induced nicotine accumulation and compared the nicotine-inducing ability of exogeneous additions of linolenic acid (18:3) and its methyl ester, linoleic acid (18:2), abscisic acid, traumatic acid, and methyl dihydrojasmonate to the nicotine-inducing ability of exogenous additions of methyl jasmonate (MJ). The first four of these inhibitors significantly reduced wound-induced nicotine accumulation when applied in a lanolin paste to wounded tissues immediately after wounding at concentrations of 89–90 μg/plant. When methyl salicylate and propyl gallate were mixed individually with MJ, neither inhibited MJ-induced nicotine synthesis, which suggests that the inhibitors block jasmonate synthesis or release from stored pools and not its effects. Linolenic acid or its methyl ester applied to undamaged plants or damaged plants (to either damaged or undamaged leaves) or to the roots of hydroponically growing plants did not induce nicotine accumulation or increase nicotine accumulation above levels found in damaged plants. Similarly, traumatic acid, linoleic acid, and abscisic acid did not induce nicotine accumulations. Methyl dihydrojasmonate, which is biosynthetically derived from linoleic acid, had 12–56% of the nicotine-inducing acitivity of MJ when added to the roots of hydroponically grown plants. The signal transduction pathway mediating wound-induced nicotine production therefore shares many features of the pathway eliciting wound-induced proteinase inhibitor production but differs in not being regulated at the lipase step in jasmonic acid production and not being responsive to abscisic acid.

Key Words—Induced defense, *Nicotiana sylvestris*, nicotine, methyl jasmonate, linolenic acid, abscisic acid, methyl dihydrojasmonate.

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INTRODUCTION

Nicotiana sylvestris is an annual that accumulates nicotine at 0.1–1% leaf dry mass in undamaged plants, but after real or simulated folivory, nicotine concentrations in remaining leaves increase dramatically to attain concentrations (1–4%) that are sufficient to protect induced tissues from nicotine-adapted insect herbivores in laboratory feeding trials (Baldwin, 1988a–c, 1989, 1991). The induced increase in whole-plant nicotine pools is thought to result primarily from increases in *de novo* synthesis. Leaf damage results in threefold increases in the rates of *de novo* nicotine synthesis as determined with ^{15}N pulse-chase techniques (Baldwin et al., 1994b). Diverse experiments indicate that nicotine is synthesized largely in the roots and transported to the shoot in the xylem stream. For example, when the roots and shoots of nicotine- and non-nicotine-producing species are grafted together in different combinations, only the combinations with nicotine-producing roots accumulate nicotine in substantial quantities (Dawson, 1942; Baldwin, 1991). Leaf damage dramatically increases the amount of nicotine exported from the roots in the xylem stream of tobacco plants (Baldwin, 1989; Baldwin et al., 1993). Induced increases in nicotine accumulation are inhibited in plants whose stems have been steam girdled below the leaf damage (Baldwin, 1989). Last, the distribution of nicotine's biosynthetic enzymes is consistent with nicotine synthesis in roots. Wagner et al. (1986) and Feth et al. (1986) determined that putrescine *N*-methyltransferase (PMT) was the key regulatory enzyme in nicotine biosynthesis, that roots had higher PMT activity than shoots, and that PMT activity was stimulated by damage to the shoot (Mizusaki et al., 1973; Saunders and Bush, 1979; Wagner et al., 1986). The sequence of the PMT gene from *N. tobaccum* has recently been determined: the mRNA coding for the PMT is found only in root tissues and the amount of mRNA increases after damage to the shoot (Hibi et al., 1994).

This large separation between the site of nicotine synthesis and the site of herbivore attack requires a long-distance signal transduction pathway, and some advances have been made in understanding this signal transduction pathway. The damage cue is a positive cue and correlates with the amount of damage a plant receives (Baldwin, 1989; Baldwin and Schmelz, 1994). The fact that it can be blocked by steam girdling the stem of a plant below the site of damage suggests that it is phloem-borne: steam kills the phloem but leaves the xylem intact. The damage signal is relatively slow-moving, taking more than 1 hr to leave a damaged leaf. This makes an electrical damage signal unlikely (Baldwin et al., 1994a). Because the *de novo* rates of nicotine synthesis increase 10 hr after leaf damage (Baldwin et al. 1994a,b), the signal that effects these changes in root nicotine metabolism must travel from damaged leaves to the roots within this time period. Jasmonic acid (JA) is intimately involved in the signal transduction cascade, because leaf damage rapidly (<0.5 hr) increases shoot JA

pools and, more slowly (<2 hr), root JA pools. JA levels subsequently decay to levels found in undamaged plants within 24 hr and 10 hr for shoots and roots, respectively. Moreover, additions of JA to roots as its methyl ester, MJ, increase *de novo* nicotine synthesis from $^{15}NO_3$ in a manner similar to that induced by leaf damage; and additions of MJ to the shoots mimic both the timing of changes in pool size induced by damage and the magnitude of increased nicotine synthesis. It is not yet known if the wound-induced JA in the shoot is directly transported to the roots or if some other indirect signal pathway is involved. In tomato, the octadecapeptide, systemin, stimulates the wound-induced increase in proteinase inhibitors (PIs) by increasing the pools of JA (Farmer et al., 1994). A similar transduction pathway may be also be responsible for wound-induced nicotine production. Hence, the working model for the long-distance signal transduction cascade is that wounding increases the shoot pool of jasmonates, which, either directly through transport or indirectly through a systemin-like signal, increases the jasmonate pool in the roots; these, in turn, stimulate root nicotine synthesis and increase whole-plant nicotine pools (Figure 1).

The signal transduction pathways responsible for inducing PIs in tomato and potato and of benzo[*c*]phenanthridine alkaloids in tissue culture are better understood than the wound-induced production of nicotine in tobacco in four important regards. First, in the PI system, the addition of mammalian cyclooxygenase (COX) and lypoxygenase (LOX) inhibitors to wounded tissues, which are thought to inhibit lypoxygenases or hydroperoxide dehydrases in the biosynthesis of JA from linolenic acid (LA), inhibit induced PI production and inhibit the wound-induced increase in jasmoantes (Peña-Cortés et al., 1993, 1995). Moreover, the addition of diethyldithiocarbamic acid (DIECA), which reduces 13-hydroperoxylinolenic acid to the hydroxylinolenic acid and thereby scavenges substrates from the jasmonate biosynthetic pathway, inhibits both the wound- and systemin-induced increase in PIs (Farmer et al., 1994). These results demonstrate that jasmonate synthesis from LA to the production of 12-oxophytodienoic acid (Figure 1) is a necessary requirement for wound-induced PI production. Second, in the PI system (Farmer and Ryan, 1992), as well as in other jasmonate-induced responses, such as the tendril-coiling response of *Bryonia dioica* (Falkenstein et al., 1991), the induced responses appear to be regulated at the lipase step in the biosynthesis of jasmonates because the addition of exogenous LA to plants induces the responses. Third, the common plant hormone, abscisic acid (ABA) activates the wound-induced production of PI II in tomato and potato (Hildmann et al., 1992; Peña-Cortés et al., 1995). Fourth, a second lipid cascade starting with linoleic acid via 15, 16-dihydro-12-oxophytodienoic acid to 9,10-dihydrojasmonic acid (dhJA) is also active in the induction of the enzymes in the benzo[*c*]phenanthridine alkaloid biosynthetic pathway in tissue culture (Blechert et al., 1995). In these well-described model systems, wounding, signal production, and response occur in the same tissues. Without

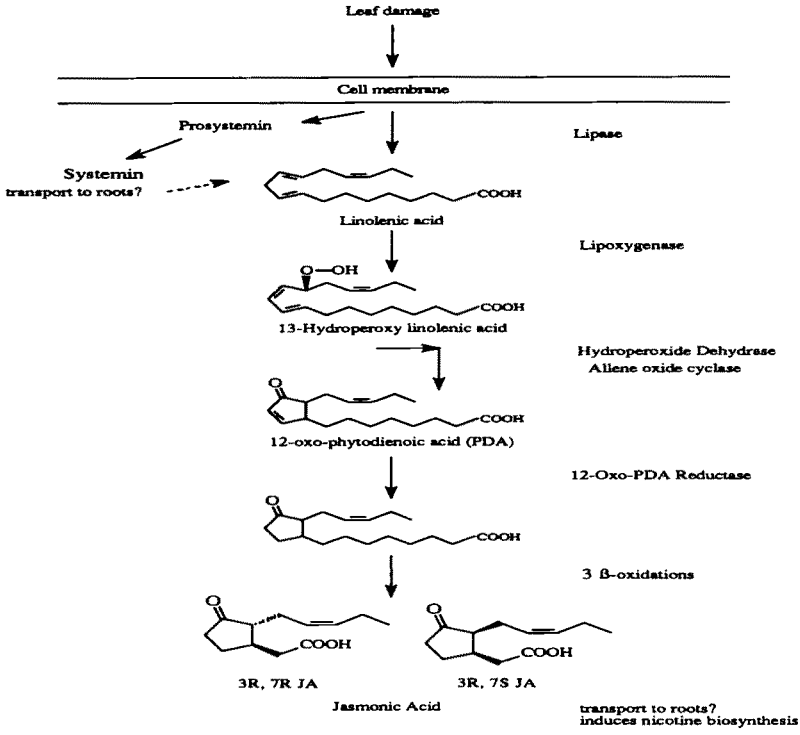


FIG. 1. Biosynthesis of jasmonic acid (JA). For the nicotine system, we have established that leaf damage increases endogenous pools of JA first in the shoots and 2 hr later in the roots (Baldwin et al., 1994a). The stereoconfiguration of carbon-3 and -7 of the induced JA pool remains unresolved; the two naturally occurring isomers are depicted. Exogeneous addition of methyl jasmonate to leaves mimics both the wound-induced changes in JA in the root and shoot and the changes in nicotine pools.

a large spatial separation between the sites of damage and the wound-induced responses, local and systemic transduction pathways are difficult to untangle.

Here we explore these four aspects of the signal transduction pathway mediating wound-induced nicotine production. Specifically, we report experiments that examine the effect of: (1) mammalian LOX and COX inhibitors on wound- and jasmonate-induced nicotine accumulation; and (2) exogenous additions of LA and its methyl ester (Me-LA), linoleic acid, ABA, traumatic acid, and methyl dihydrojasmonate (dhMJ) on induced nicotine accumulation. We examine the effect of traumatic acid, because it is a derivative of 13-hydroperoxy

linolenic acid, which, to our knowledge, has not been examined in any wound-induced defense response. Traumatic acid is known to stimulate leaf abscission in cotton (Strong and Kruitwagen, 1967) but has largely been ignored in the recent surge of interest in the function of octadecanoid metabolites (Farmer, 1994).

METHODS AND MATERIALS

Chemicals. MJ and dhMJ were obtained from Bedoukian Research (Danbury, Connecticut). We determined by GC-MS analysis that MJ was close to the thermodynamic equilibrium for the epimers of MJ (Mueller and Brodschelm, 1994): 90.1% 1*R*,2*R* MJ and 8.3% 1*R*,2*S* MJ. The dhMJ contained 98.14% 1*R*,2*R* dhMJ and 1.86% 1*R*,2*S* dhMJ and did not contain detectable quantities of MJ. All plant nutrients and other chemicals used were purchased from Sigma Chemical Co. (St. Louis, Missouri); oxygen-sensitive compounds were stored under nitrogen at −20°C.

Plant Growth. We report the results of eight experiments. The plants used in each experiment were grown in hydroponic culture with the following protocol: *Nicotiana sylvestris* Spegazzini and Comes seeds were germinated and grown in Cornell mix A (Boodley and Sheldrake, 1977) for 15–20 days, after which they were transferred to 40-liter hydroponic boxes containing a complete nutrient solution [0.292g/liter Peter's Hydro-sol (W.R. Grace, Inc., Fogelsville, Pennsylvania), 0.193g/liter $CaNO_3$, and 70m/liter of a nitrogen-free nutrient solution (described in Ohnmeiss and Baldwin, 1994)] for three to four days. To reduce between-plant genetic variance, we used seeds that were full sibs. Plants were transferred to individual opaque 1-liter containers with the nitrogen-free nutrient solution that was supplemented with KNO_3 to produce a 2.0 mM solution. Solutions in the 1-liter containers were maintained at 900–1000 ml with additions of the no-nitrogen nutrient solution. All plants were grown in a glasshouse with supplemental lighting from 400-W sodium-vapor lamps for 16 hr/day, which provided a minimum PAR of 220 μmol photons m^{-2} s^{-1}. After 8–12 days of growth in the individual containers, plants were weighed. Plants with the most similar masses and morphologies were assigned to treatment groups of five plants each, sorted according to wet mass, and assigned to treatment groups by consecutive random divisions (as described in Ohnmeiss and Baldwin, 1994). This consecutive random allocation of plants produced groups with plant wet mass means that were not significantly different on day 0, the start of each experiment. Plants were randomly assigned to positions on the glasshouse bench independently of their treatment. The nitrogen-free nutrient solution of each individual plant chamber was supplemented with KNO_3 to produce a 0.5 mM solution on day 0, and all plants were harvested on day 5,

the time when maximum damage-induced nicotine production had occurred after a single damage event (Baldwin et al., 1994b).

Leaf Damage. Reproducible amounts of leaf damage were produced by rolling a pattern-tracing wheel (Dritz Spartanburg, South Carolina) across two leaves at phylotactically adjacent positions 3 and 4 (with the apical leaf designated as position 1). Each leaf received eight rows of evenly distributed tracing-wheel damage on each side of the mid-rib over a 4-cm length of the lamina in the middle of the lamina. On average, tracing-wheel damage resulted in 4.5 1-mm^2 punctures/cm of leaf and killed the cells in the puncture zone. Punctures were produced by crushing leaf tissue between the blunt spokes of the tracing wheel and a plastic card, and resulted in minimal vasculature damage and undetectable leaf dry mass loss or reductions in biomass gain (Ohnmeiss and Baldwin, 1994; Baldwin and Schmelz, 1994). In all experiments in which leaves were treated with compounds in a lanolin paste, lanolin was spread on all plants in the area to be damaged and the tracing wheel was rolled over the area treated with lanolin.

Application of Chemicals to Plants. Chemicals were applied to plants in either lanolin pastes on leaves or in aqueous solutions applied in the hydroponic growth medium. Lanolin pastes were applied to the adaxial surface of leaves occupying positions 3 and 4 from the apical leaf. The lanolin (0.093 $\pm$ 0.001 g/plant) was spread with a plastic applicator in a thin layer spanning the middle 4 cm of the lamina and was divided equally between the two leaf positions. Control untreated plants and damaged plants had the same leaf positions treated with the same amount of pure lanolin. Lanolin pastes, containing precisely quantified amounts of the chemicals to be added to plants, were prepared less than 2 hr before their application. They were prepared by liquefying lanolin in a warm (50°C) water bath, adding the chemical, stirring vigorously, and weighing (on wax paper) aliquots to be added to each plant. The amount of chemicals added to each plant is presented (micrograms) in the figure captions.

Whole-Plant Nicotine Concentrations. On day 5 of each experiment, plants were separated into roots and shoots, frozen in liquid nitrogen, lyophilized, weighed (to 0.1 mg), and ground to a fine powder (850 μm mesh) in a Wiley mill (Thomas Scientific, Swedesboro, New Jersey). Nicotine analysis was modified from Baldwin (1988c) to obtain whole-plant nicotine measures. A weighed portion (approximately 50 mg) of lyophilized and homogenized plant was extracted for three days in 10.0 ml of aqueous alkaloid extraction solution (40% methanol, 0.1% HCl). Nicotine contents of these extracts were determined by HPLC with external standard curves run with each experiment. Whole-plant nicotine concentrations were calculated from biomass values and whole-plant nicotine pools.

Statistical Analysis. Nicotine concentrations were arcsine-transformed before statistical analysis. One-way ANOVAs with treatments as main effects

were performed for biomass and nicotine concentration parameters, and contrasts among treatment groups were constructed from the one-way ANOVAs. All data analyses were performed with the MGLH module of the SYSTAT statistical package (Evanston, Illinois).

RESULTS

Effects of Inhibitors. Three inhibitors (ibuprofin, mefenamic acid, and DIECA applied at 90, 98, and 93 μg/plant, respectively) killed cells in the leaf five days after the lanolin paste containing these compounds were applied. The damage caused by these inhibitors likely overwhelmed any effect the inhibitors might have had on the mechanical wounding of the leaf, and these treatments were dropped from the analysis. Five inhibitors (Figure 2) caused no visible damage to the leaves on which they were applied, and undamaged plants treated with the same amounts of each inhibitor had nicotine concentrations not different from plants treated only with pure lanolin ($F_{1,24} = 0.234$). Wounded plants treated with pure lanolin had nicotine concentrations significantly higher than unwounded plants treated with pure lanolin ($F_{1,28} = 8.34$; $P < 0.0074$) and

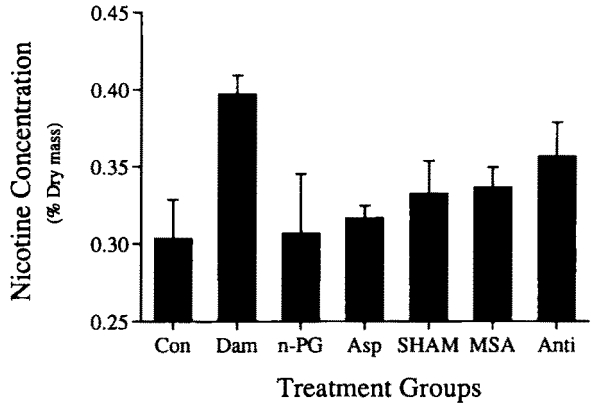


FIG. 2. Mean (±SEM) whole-plant nicotine concentrations of undamaged plants with leaves treated with pure lanolin (Con) and plants damaged with a tracing wheel where the damaged areas of the leaves were thinly coated with either pure lanolin (Dam) or lanolin containing 89–91 μg/plant of the following mammalian LOX and COX inhibitors: *n*-propyl gallate (*n*-PG); acetosalicylic acid (Asp); salicylhydroxamic acid (SHAM); methyl salicylate (MSA); and antipyrine (Anti). Plants were harvested for alkaloid analysis five days after treatment applications.

wounded plants treated with 89–90 μg/plant of either propyl gallate (*n*-PG), aspirin, SHAM, or methyl salicylate (MSA) ($F_{1,28} = 8.10$; $P < 0.0082$). Wounded plants treated with the latter four inhibitors had nicotine concentrations not significantly different from unwounded plants ($F_{1,28} = 0.651$; $P < 0.426$). Wounded plants treated with 91 μg/plant of antipyrine had nicotine concentrations not sigificantly different from wounded plants ($F_{1,28} = 1.44$; $P < 0.239$).

In a second experiment (Figure 3), the ability of two inhibitors (*n*-PG and MSA) to inhibit both wound- and MJ-induced responses were examined. Again, wounding significantly increased nicotine concentrations above unwounded controls ($F_{1,28} = 12.06$; $P < 0.0001$), and the two inhibitors applied at 90 μg/plant significantly suppressed the wound-induced nicotine accumulation ($F_{1,28} = 4.42$; $P < 0.002$). Unwounded plants treated with 90 μg/plant of MJ mixed with an equal amount of either MSA or *n*-PG had nicotine concentrations not

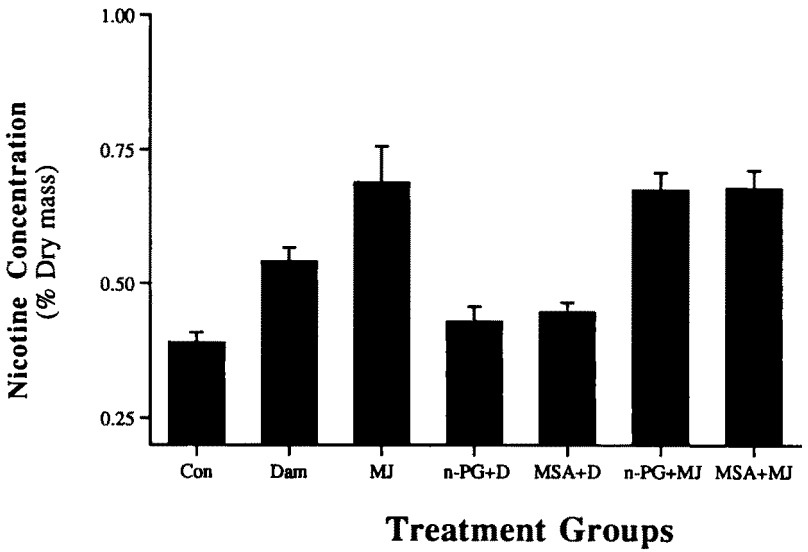


FIG. 3. Mean (±SEM) whole-plant nicotine concentrations of undamaged plants with leaves treated with pure lanolin (Con), lanolin containing 90 μg/plant of *n*-propyl gallate and 90 μg/plant of methyl jasmonate (*n*-PG + MJ), or 90 μg/plant of methyl salicylate and 90 μg/plant of methyl jasmonate (MSA + MJ). Plants also were damaged with a tracing wheel and the damaged areas of the leaves were thinly coated with either pure lanolin (Dam), lanolin containing 90 μg/plant of *n*-propyl gallate (*n*-PG + D), or methyl salicylate (MSA + D). Plants were harvested for alkaloid analysis five days after treatment applications.

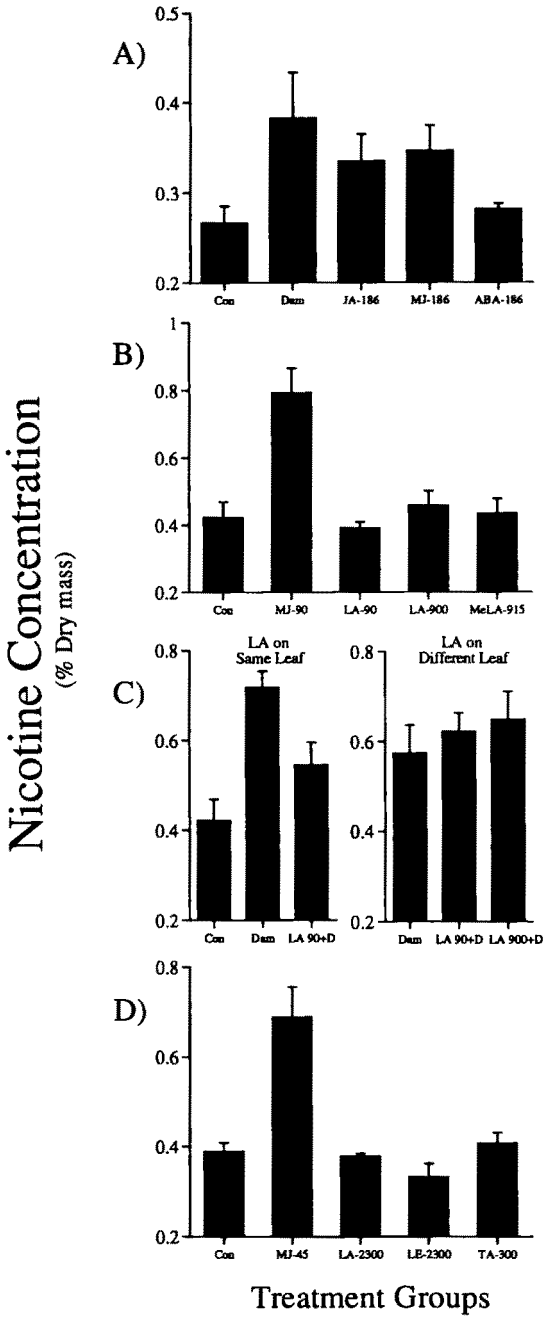
significantly different from those treated with MJ alone ($F_{1,28} = 0.019$ and 0.037, respectively). These results demonstrate that putative LOX or hydroperoxide dehydrase inhibitors can quantitatively suppress a wound-induced response but do not suppress MJ-induced increases in nicotine at the applied concentrations.

Effects of Fatty Acids, Abscisic Acid and Traumatic Acid. Abscisic acid (ABA), MJ, and JA were applied to unwounded plants at 186 μg/plant (Figure 4A). This was the highest concentration of ABA that did not significantly inhibit growth five days after application as determined from a preliminary experiment. JA, MJ, and wounding significantly increased nicotine concentrations above unwounded controls ($F_{1,26} = 5.03$–7.84; $P < 0.0034$). The nicotine concentration of plants treated with ABA did not differ from controls ($F_{1,26} = 0.19$).

The nicotine concentration of unwounded plants treated with 90, 900, and 2300 μg/plant LA (Figure 4B and D) or with 915 μg/plant of the methyl ester of LA (Me-LA; Figure 4B) applied to their leaves did not differ significantly ($F_{1,20} = 0.0223$ and $F_{1,12} = 1.85$) from control plants treated only with lanolin. Similarly, wounded plants with LA applied to phylotactically adjacent unwounded leaves at position 5 had nicotine concentrations (Figure 4C) not significantly different from wounded controls ($F_{1,24} = 1.02$). Wounded plants treated with LA directly to the wounds (Figure 4B) had nicotine concentrations lower than wounded controls ($F_{1,24} = 5.35$; $P = 0.029$) but higher than unwounded controls ($F_{1,24} = 4.58$). Plants treated with traumatic acid at 300 μg/plant or linoleic acid at 2300 μg/plant (Figure 4D) had nicotine concentrations not sigificantly different from control plants ($F_{1,28} = 0.21$). Nicotine production was therefore not induced by the addition of fatty acids, ABA, or traumatic acid to leaves in lanolin pastes in a manner comparable to nicotine inductions resulting from the addition of MJ or JA in lanolin pastes. Experiments with the addition of LA in DMSO, detergents, or directly without a carrier to leaves caused substantial amounts of cell death at the site of application and therefore could not be used.

To test the hypothesis that failure of LA to induce nicotine synthesis might be because it is not transported from sites of wounding in the shoot to sites of nicotine synthesis in the roots, we added Me-LA to the roots of hydroponically growing plants. Additions of Me-LA at 10, 100, or 200 μg/plant did not affect plant growth or nicotine concentrations (Figure 5: $F_{1,60} < 0.14$). The addition of LA to the roots at 10 μg/plant inhibited growth in a preliminary test and therefore the free fatty acid could not be used.

MJ and dhMJ Comparison. MJ was found to be significantly more active in inducing plants than dhMJ at all concentrations tested (Figure 5). At 10 μg/plant dhMJ was 12.5% as active as MJ; at 50 μg/plant, 33.6%; at 100 μg/plant, 42.2%; and at 200 μg/plant, 56.4% as active.


Nicotine Concentration
(% Dry mass)
A)
0.5
0.4
0.3
0.2
Con
Dam
JA-186
MJ-186
ABA-186
B)
1
0.8
0.6
0.4
0.2
Con
MJ-90
LA-90
LA-900
MeLA-915
C)
LA on Same Leaf
LA on Different Leaf
0.8
0.6
0.4
0.2
Con
Dam
LA 90+D
Dam
LA 90+D
LA 900+D
D)
0.8
0.6
0.4
0.2
Con
MJ-45
LA-2300
LE-2300
TA-300
Treatment Groups

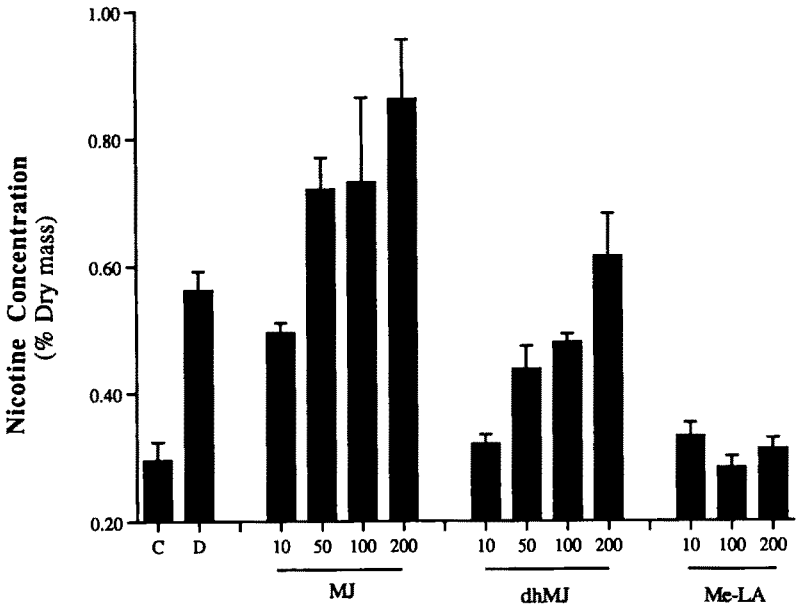


FIG. 5. Mean (±SEM) whole-plant nicotine concentrations of undamaged plants (C), plants damaged with a tracing wheel (D), and undamaged plants that had 10, 50, 100, or 200 μg/plant of methyl jasmonate (MJ) or methyl dihydrojasmonate (dhMJ) or 10, 100, or 200 μg/plant of linolenic acid methyl ester (Me-LA) added to the hydroponic solution of each plant. Plants were harvested for alkaloid analysis five days after treatment applications.

FIG. 4. Mean (+SEM) whole-plant nicotine concentrations of undamaged plants with leaves treated with pure lanolin (Con); plants damaged with a tracing wheel where the damaged leaf areas were thinly coated with either pure lanolin (Dam) or lanolin containing 90 μg/plant of linolenic acid (LA 90 + D); and undamaged plants each treated with lanolin containing 186 μg of jasmonic acid (JA-186), 186, 90, or 45 μg of methyl jasmonate (MJ-186, MJ-90, MJ-45), 186 μg of abscisic acid (ABA-186), 90, 900, or 2300 μg of linolenic acid (LA-90, LA-900, LA-2300), 915 μg of linoleic acid methyl ester (MeLA-915), 2300 μg of linoleic acid (LE-2300), or 300 μg of traumatic acid (TA-300). For all of the above treatments, damage and lanolin applications were performed on leaves in positions 3 and 4 from the apical leaf. For treatments in panel C labeled "LA on Different Leaf," leaf position 4 was damaged and lanolin containing either 90 μg (LA 90 + D) or 900 μg (LA 900 + D) of linolenic acid was applied to the leaf in position 3 of each plant. Plants were harvested for alkaloid analysis five days after treatment applications.

DISCUSSION

Mammalian LOX and COX inhibitors inhibit the wound-induced accumulation of nicotine when applied to the wound site but are not able to inhibit MJ-induced accumulations (Figures 2 and 3). These results are consistent with the requirement of wound-induced jasmonate synthesis for nicotine induction and suggest that the inhibitors function by inhibiting steps upstream of JA synthesis in the octadecanoid pathway. These results are similar to those found in the PI system (Peña-Cortés et al., 1993, 1995) but should be interpreted cautiously. The effect of inhibitors on jasmonate pools in damaged leaves and on their transport to the roots needs to be examined before we can conclude that wound-induced jasmonate synthesis is a necessary step in the pathway leading to wound-induced nicotine production.

While the results of the inhibitor experiments highlight similarities between the induced-nicotine and -PI systems, the results of the fatty acid addition experiments (Figures 4 and 5) demonstrate that the signal cascade activating induced nicotine production differs subtly from that responsible for the induction of PIs in two regards. First, the plant hormone, ABA, which induces PI II genes (Hildmann et al., 1992), does not induce nicotine production in concentrations (300 μg/plant) 10 times that required to induce nicotine production with MJ or JA (Baldwin et al., 1994a) (Figure 5). Second, the exogenous addition of LA, the 18:3 fatty acid precursor of JA (Figure 1), or its methyl ester to leaves does not induce nicotine production, as it clearly does in the PI system (Farmer and Ryan, 1992) or in the tendril-coiling response of *Bryonia* (Falkenstein et al., 1991). One way to reconcile the differences in effects of LA on the different systems is that in the PI system, PIs are produced in the shoot and adding LA may increase local JA production and local PI synthesis. In the nicotine system—where the response occurs primarily in the distal tissues, namely the roots—adding LA may indeed induce JA synthesis locally. If JA is not transported to the roots, however, it would not affect nicotine synthesis. This possible reconciliation was clearly falsified by the addition of the fatty acid to the roots (Figure 5), where it was found to be inactive. We found that the addition of LA at high concentrations in lanolin pastes or detergents caused extensive cell death at the application site, and it is possible that the activity of LA in some tests may be due to "wounding." More plausible is the possibility that in the PI system, inducible JA synthesis is regulated at the lipase step and at some point later in the pathway in the nicotine system.

The results presented here also demonstrate that dhMJ, the product of the parallel octadecanoid pathway starting from the related 18:2 fatty acid, linoleic acid, was active in inducing nicotine synthesis, but only 12–56% as active as MJ in inducing nicotine synthesis. dhMJ is also less active than MJ in inducing the enzymes of the benzo[*c*]phenanthridine alkaloid biosynthetic pathway

(Blechert et al., 1995). Since the supply of MJ and dhMJ used in these experiment differed slightly in the ratio of their respective 1*R*,2*R* and 1*R*,2*S* epimers, the possibility exists that the difference in activities is due to the difference in epimers. We find this possibility unlikely because we have found no difference in the nicotine-inducing activity of the two naturally occurring epimers of JA (Figure 1) under experimental conditions similar to those reported here (Zhang and Baldwin, unpublished results). Since the exogeneous addition of linoleic acid, like the addition of LA, also failed to induce nicotine accumulations (Figure 4D), it appears that the pathway leading to dhJA synthesis is also regulated after the lipase step. This conclusion is contingent on the demonstration that dhJA pools are induced by damage.

The octadecanoid pathway in plants, like the prostaglandin pathway in animals, elicits a diverse suite of responses, many of which relate to wounding and microbial attack. The fact that the plant pathway can be inhibited by LOX and COX inhibitors used by humans to suppress the pain and inflammation mediated by prostaglandins in response to wounding may prove to be an important tool for chemical ecologists interested in the functional significance of induced responses.

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IDENTIFICATION OF SEX PHEROMONE OF *Tetanolita mynesalis* (LEPIDOPTERA: NOCTUIDAE), A PREY SPECIES OF BOLAS SPIDER, *Mastophora hutchinsoni*

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Abstract—The bolas spider, *Mastophora hutchinsoni*, attracts adult males of four species of nocturnally active Lepidoptera through aggressive chemical mimicry of those species' sex pheromones. Here we report the identification of the sex pheromone of one prey species, the smoky tetanolita (*Tetanolita mynesalis*). In sex pheromone gland extracts, only two peaks stimulated an electrophysiological response as measured by a coupled gas chromatography–electroantennographic detection analysis. These two peaks had retention times identical to (3*Z*,6*Z*,9*Z*)-heneicosatriene (3*Z*,6*Z*,9*Z*-21:H) and (3*Z*,9*Z*)-*cis*-6,7-epoxy-heneicosadiene (3*Z*,9*Z*-*cis*-6,7-epoxy-21:H), respectively, and mass spectra identical to these two compounds. It was determined that 0.23 ± 0.16 and 0.56 ± 0.26 ng of 3*Z*,6*Z*,9*Z*-21:H and 3*Z*,9*Z*-*cis*-6,7-epoxy-21:H, respectively, were present in pheromone gland extracts from individual females. A 1:1 blend of 3*Z*,6*Z*,9*Z*-21:H and 3*Z*,9*Z*-6*S*,7*R*-epoxy-21:H was an effective attractant for adult males from feral populations. Blend ratios of these two components from 2:1 to 1:2 were equally effective as attractants. Greater deviation from the optimal blends resulted in diminished trap catches. The enantiomer 3*Z*,9*Z*-6*R*,7*S*-epoxy-21:H not only was not effective in attracting males, its presence in the effective blend shut down trap catches. These results indicate that the pheromone blend consists of 3*Z*,6*Z*,9*Z*-21:H and 3*Z*,9*Z*-6*S*,7*R*-epoxy-21:H. This is the first report of a hydrocarbon/epoxide pheromone for a prey species of this bolas spider. Sex attractants or pheromones for the other three prey species are composed of aldehydes or acetates.

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Key Words—Chemical mimicry, pheromone, *Mastophora hutchinsoni*, bolas spider, *Tetanolita mynesalis*, 3*Z*,6*Z*,9*Z*-heneicosatriene, 3*Z*,9*Z*-6*S*,7*R*-epoxyheneicosadiene.

INTRODUCTION

Late-stadia and adult females of bolas spiders in the genus *Mastophora* hunt by aggressive chemical mimicry of the sex pheromones of their lepidopterous prey (Eberhard, 1977; Stowe et al., 1987; Yeargan, 1988, 1994). Only adult males of a limited subset of available prey species are attracted to volatile chemicals emitted by these spiders (Stowe, 1988; Yeargan, 1994). The bolas spider, *Mastophora hutchinsoni* Gertsch, has been found to capture only four species of nocturnally active Lepidoptera, including three noctuid moths—the smoky tetanolita, *Tetanolita mynesalis* (Walker), the bristly cutworm, *Lacinipolia renigera* (Stephens), and the bronzed cutworm, *Nephelodes minians* Guenée—and one pyralid moth, the bluegrass webworm, *Parapediasia teterrella* (Zincken) (Yeargan, 1988). Sex pheromones or attractants for the latter three species have been shown to be unsaturated long-chain aliphatic acetates or aldehydes (Clark and Haynes, 1990; Haynes, 1990; Underhill et al., 1977). There are no known attractants or pheromone components for *T. mynesalis*. However, this species belongs to the Herminiinae, a subfamily of Noctuidae within which monoepoxide derivatives of triene or diene hydrocarbons have been shown to be attractants for several species (Wong et al., 1985; Millar et al., 1991). This information concerning the known sex pheromones and attractants for three of the prey species, and phylogenetic inferences regarding potential attractants for one of the most frequently captured prey species, *T. mynesalis*, suggested that this bolas spider may be capable of producing a biosynthetically diverse set of allomones. The objective of the research described herein was to identify the female-produced sex attractant pheromone of *T. mynesalis*, thus providing additional indirect evidence that addresses this hypothesis.

METHODS AND MATERIALS

Insects. Adult *T. mynesalis* were collected at blacklight traps in suburban backyards in Lexington and Richmond, Kentucky, during June–August 1994. Captured adults were transferred to 473-ml paper cartons with nylon screen lids. Eggs were removed from the inner surface of the carton and transferred to a 10-cm-diam. × 1.5-cm Petri dish containing a damp 7.5-cm piece of filter paper. Hatched larvae were transferred to plastic containers (15 cm diam. × 7.5 cm) lined with moist paper towels containing a mulch consisting primarily

of white oak, *Quercus alba* L., leaves that had fallen to the ground the previous autumn. Covell (1984) indicated the larval food for *T. mynesalis* was probably dead leaves. With this suggestion as our starting point, we selected white oak leaves as the larval food when these leaves proved to be adequate to rear *T. mynesalis* to the adult stage. Larvae were reared under laboratory conditions, without any effort to control light or temperature cycles. After pupation, male and female pupae were separated by sex based on external genital characteristics (Mosher, 1969). Pupae were kept in Petri dishes with moist filter paper. On emergence, adults were transferred to 473-ml paper cartons. Pupae and adults were held in an environmental chamber with a 16L:8D light cycle and temperature cycle of 25°C:18°C.

Chemicals. The *n*-pentadecane and *n*-tricosane used as internal standards were obtained from Alltech (Deerfield, Illinois). Z9-14:Ac and Z11-16:Al, which were used in standard solutions for GC-EAD, were obtained from the Institute of Pesticide Research (Wageningen, The Netherlands) and Scentry (Buckeye, Arizona), respectively. 3*Z*,9*Z*-6*S*,7*R*-Epoxy-21:H (94.15% enantiomeric purity, or 88.3% enantiomeric excess) and 3*Z*,9*Z*-6*R*,7*S*-epoxy-21:H (94.10% enantiomeric purity, or 88.2% enantiomeric excess) were synthesized previously (Millar and Underhill, 1986). 3*Z*,6*Z*-9*Z*-21:H was 96.4% pure and was synthesized as described by Underhill et al. (1983).

Extraction of Pheromone Glands. When females were 2–5 days old (primarily 3–4 days old), they were removed from the environmental chamber at midscotophase. Gentle pressure was applied to the abdomen of each female until the ovipositor and the associated pheromone gland emerged. The ovipositor and gland were removed with a pair of fine dissecting scissors, and placed into a 100-μl conical-bottom vial containing 20 μl of methylene chloride. For pooled extracts, several glands were extracted per vial. For analyses intended to document variation in pheromone content among individuals, only one gland was extracted per vial. Each vial was either analyzed immediately or sealed and stored at −80°C until needed. Before analysis, a vial was removed from the freezer and allowed to warm to room temperature. For the study of individual variation, 10 μl of an internal standard solution containing 1 ng/μl of *n*-pentadecane (15:H) and *n*-tricosane (23:H) were added to each vial. Previously it was determined that these internal standards would not coelute with components of the gland extracts. The volume of the solution was reduced under N_2 to 1 to 2 μl before injection.

Chemical and Electrophysiological Analysis. The GC-electroantennographic detection system was similar to that described by Struble and Arn (1984) and Leal et al. (1992). A DB-5 column (30 m × 0.25 mm ID, J&W Scientific Folsom, California) was installed in a Hewlett-Packard 5890 Series II GC. Injections were splitless. The effluent from the column was split at a 1:1 ratio between a flame ionization detector (FID) and a electroantennographic detector

(EAD). The oven temperature was held at 80°C for 2 min and then increased to 260°C at a rate of 20°C/min. Just prior to the split, a makeup gas (nitrogen) flow rate of 120 ml/min was introduced via a VSIS-5 T connector (Scientific Glass Engineering, Austin, Texas). The effluent flowed out of this T connector through a 5-cm section of deactivated column (0.53 mm), which was inserted into a deactivated Universal Y Press-Tight connector (Resteck, Bellefonte, Pennsylvania). A 10-cm section of deactivated column was used to make a connection of one branch of the Y to the FID. An identical section of column connected the other branch of the Y through a separate heated zone into the side of a modified glass condenser. Air flowed through the system at 1 liter/min and was humidified by bubbling through water before it entered the condensor. Chilled water circulated through the outer jacket of the condenser, cooling the humidified air to 22°C. This air flowed over the end of the GC column and delivered the GC effluent to the antennal preparation, which was 5 cm downstream from the end of the GC column.

The antennal preparation was similar to that of Leal et al. (1992) with the following exceptions. The base of the moth's excised head was placed into a drop of Pringle's saline (Pringle, 1938). The terminal segment of one antenna was removed, and the cut tip placed into a separate drop of saline. Silver–silver chloride electrodes were placed into each drop of saline and connected through a Grass P16 high-impedance probe to a Grass P16 amplifier (Quincy, Massachusetts). Baseline drift was controlled by the passive high-pass filter (capacitor = 10,000 μF, resistor = 130 Ω) described by Struble and Arn (1984). The amplified EAD and FID signals were sent to a Dataq model DI-420 Signal Conditioning Module (Akron, Ohio). Both signals were recorded and analyzed using Dataq MCA-CODAS software (Akron, Ohio).

A Hewlett-Packard 5890 Series II gas chromatograph equipped with either a DB-5 or a DB-WAX column (both columns 30 m × 0.25 mm ID, J&W Scientific, Folsom, California), linked with a Hewlett-Packard 5972 mass selective detector (MSD), was used for analysis of gland extracts. GC operating conditions for both columns were the same with an initial oven temperature of 50°C for 2 min, increasing to 240°C at a rate of 10°C/min. The flow rate of helium through the column was maintained at 1 ml/min. The MSD was set to scan from 40 to 400 amu, to obtain electron impact (70 eV) spectra.

Field Tests. For the first field exp riment, the quantity of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21:H applied to a red rubber septum (A.H. Thomas, Swedesboro, New Jersey) that had previously been extracted two times in hexane was kept constant at 100 μg and the quantity of 3*Z*,6*Z*,9*Z*-21:H was varied to yield 1:1, 4:1, and 10:1 blend ratios of this epoxide relative to the hydrocarbon. Fifty microliters of one of these three blends in hexane or hexane only was loaded onto a septum. Each rubber septum was hung by a paper clip from inside the top of a Pherocon IC trap (Trécé, Salinas, California). These traps were suspended from

limbs of trees so that the trap opening was approximately 1.8 m above the ground, which corresponds to a normal height at which the bolas spider *M. hutchinsoni* has been observed to catch male *T. mynesalis*. Traps were separated by at least 5 m. The assignment of a particular treatment to one of these trap positions was determined at random. The positions of treatments were rerandomized after each trapping period (one or two nights). A priori we had decided not to include any blocks of the experiment in which no males were caught in any of the four treatments (unsuccessful replications). A total of 13 successful replications were conducted between 23 and 28 September 1994 at the University of Kentucky's South Farm ($N = 2$), Coldstream Farm ($N = 2$), Spindletop Farm ($N = 2$), a suburban backyard in Lexington, Kentucky (backyard site 1) ($N = 2$), and suburban backyard in Richmond, Kentucky (backyard site 2) ($N = 5$). After each trapping period, sticky bottoms of the traps were returned to the laboratory and the numbers of male *T. mynesalis* were counted. A Kruskal-Wallis nonparametric anlaysis of variance was performed for this and subsequent experiments (Statistix 4.0, St. Paul Minnesota). This statistical approach was necessary because often control traps (and for some experiments other treatments) caught no moths; thus the homogeneity of variance assumption of the more powerful standard parametric analysis of variance was violated (with or without transformation). Means were separated with an experimentwise error rate set at $\alpha = 0.05$.

In a second field experiment, the following blend ratios of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H, 3*Z*,6*Z*,9*Z*-21 : H, and 3*Z*,9*Z*-6*R*,7*S*-epoxy-21 : H were tested: (a) 0 : 0 : 1, (b) 0 : 1 : 1, (c) 1 : 1 : 1, (d) 1 : 1 : 0, and (e) 1 : 0 : 0. The quantity of each compound when it was part of the blend to be applied to a rubber septum was kept at 100 μg. Control septa were loaded with hexane only. Because a methodological experiment conducted after our first experiment indicated that traps set at heights of about 30 cm were more effective in capturing male *T. mynesalis* than those set at 1.8 m (Haynes and Yeargan, unpublished data), we conducted this second and all subsequent tests at the lower height. Other details of this experiment were similar to our first experiment except that a trapping period was two or three nights. A total of eight successful replications were conducted between 3 and 12 October 1994 at the University of Kentucky's South Farm ($N = 2$), Spindletop Farm ($N = 2$), backyard site 1 ($N = 2$), and backyard site 2 ($N = 2$).

In the third field experiment, the following blend ratios of 3*Z*,9*Z*,-6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9-*Z*-21 : H were evaluated: (a) 1 : 1, (b) 1 : 4, (c) 1 : 10, and (d) 0 : 1. The quantity of 3*Z*,6*Z*,9*Z*-21 : H applied to a rubber septum was kept at 100 μg, and the quantity of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H was varied (100, 25, 10, and 0 μg). Control septa were loaded with hexane only. Other details of this experiment were similar to our second experiment, except that the Spindletop site was dropped because of the apparent low population density of

T. mynesalis found there, and one additional location was added at both the South Farm and Richmond (backyard site 2) sites. A total of 14 successful replications were conducted between 12 and 19 October 1994 at the University of Kentucky's South Farm ($N = 6$), backyard site 1 ($N = 2$), and backyard site 2 ($N = 6$).

In the last field experiment, the following blend ratios of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9*Z*-21 : H were evaluated: (a) 2 : 1, (b) 1 : 1, and (c) 1 : 2. The total quantity of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H plus 3*Z*,6*Z*,9*Z*-21 : H applied to a rubber septum was kept constant at 200 μg. Thus, for example, the 2 : 1 blend ratio consisted of 133 μg 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H plus 67 μg 3*Z*,6*Z*,9*Z*-21 : H. An additional septum was loaded with hexane only. Other details of this experiment were similar to our third experiment. A total of 14 successful replications were conducted between 19 and 26 October 1994 at the University of Kentucky's South Farm ($N = 6$), backyard site 1 ($N = 2$), and backyard site 2 ($N = 6$).

Voucher specimens of male *T. mynesalis* trapped with a 1 : 1 blend of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9*Z*-21 : H, and males reared from eggs laid by females caught in a black light trap have been deposited in the University of Kentucky, Department of Entomology, Collection.

RESULTS AND DISCUSSION

Chemical and Electrophysiological Analysis. The GC-EAD analysis indicated there were two compounds present in gland extracts of individual female *T. mynesalis* that stimulated male *T. mynesalis* antennae (Figure 1A). The EAD activity was observed at 12.00 and 13.20 min (DB-5), but there were no prominent FID peaks at these retention times. Other peaks were present in the gland extracts, but these did not stimulate an EAD response. The retention times of the straight-chain hydrocarbon standards 20 : H, 21 : H, 22 : H, and 23 : H were 11.52, 12.11, 12.77 and 13.52 min, respectively. Because monoepoxide derivatives of triene hydrocarbons have been shown to be attractants for several species in the same subfamily (Wong et al., 1985), we evaluated standard solutions of 17- to 21-carbon triene (3*Z*,6*Z*,9*Z*-17–21 : H) and 17- to 22-carbon monoepoxide derivatives (6*Z*,9*Z*-*cis*-3,4-epoxy-17 to 22 : H; 3*Z*,9*Z*-*cis*-6,7-epoxy-17 to 22 : H; and 3*Z*,6*Z*-*cis*-9,10-epoxy-17 to 22 : H). The 21-carbon hydrocarbon and epoxides gave the closest retention time matches to the EAD active compounds. Synthetic 3*Z*,6*Z*,9*Z*-21 : H and 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H had retention times identical to the EAD active compounds (12.00 and 13.20 min, respectively) and stimulated strong EAD responses (Figure 1B). Other compounds (e.g., aldehydes and acetates) that have been determined to be pheromone components or sex attractants for other prey species of *M. hutchinsoni* did not stimulate EAD activity in male *T. mynesalis* antennae (Figure 1B).

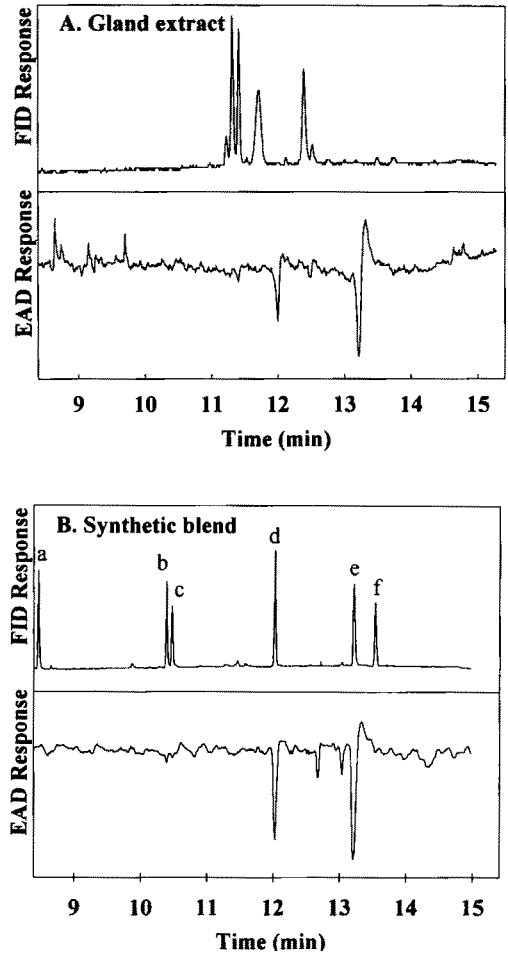

FIG. 1. (A) GC-EAD analysis of the pheromone gland extract of one female *T. mynesalis*. The top trace shows the response of the flame ionization detector (FID). The bottom trace shows the electroantennogram (EAD) response. Peaks of EAD activity were seen at retention times (R_t) of 12.00 and 13.20 min (B). A synthetic blend consisting of 0.5 ng (a) 15:H (R_t = 8.45), (b) *Z*9-14:Ac (R_t = 10.37), (c) *Z*11-16:Al (R_t = 10.45), (d) 3*Z*,6*Z*,9*Z*-21:H (R_t = 12.00), (e) 3*Z*,9*Z*-6*S*,7*R*-epoxy-21:H (R_t = 13.20), and (f) 23:H (R_t = 13.52) was analyzed by GC-EAD. The top trace shows the response of the FID. EAD responses (bottom trace) were seen at R_t of 12.00 and 13.20 min.

GC-MSD analysis of the pheromone extract using the DB-5 column indicated that two minor compounds present in individual gland extracts corresponded exactly in retention times and mass spectra with those of synthetic 3*Z*,6*Z*,9*Z*-21 : H (20.09 min) and 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H (21.66 min). From pooled extracts (5 or 10 female equivalents), the first peak and 3*Z*,6*Z*,9*Z*-21 : H were characterized by prominent ions at *m/z* 55, 67, 79, and 108, as well as less abundant ions at 234 and 290 (the molecular ion for 3*Z*,6*Z*,9*Z*-21 : H). The less abundant ions were reliably detected only in pooled extracts, while the prominent ions were consistently found in individual and pooled extracts. The diagnostic ions (Underhill et al., 1983) at *m/z* 55 ($CH_3CH_2CH{=}CH^+$) and 108 [$CH_3CH_2(CH{=}CH)_3H^+$], located the first of the three double bonds at C-3, and indicated that all three double bonds are clustered at one end of the structure. A further diagnostic ion at *m/z* 234 [$CH_3(CH_2)_{10}(CH{=}CH)_3H^+$] located a second double bond at C-9 (Underhill et al., 1983). On the basis of exact matches in retention times and spectra, and on the reasonable assumption that conjugation of the third double bond with either one of the other two would significantly change the retention time, the triene was tentatively assigned the 3*Z*,6*Z*,9*Z*-21 : H structure. Further evidence for this structure was provided by exact retention time matches of the insect-produced compound with a synthetic standard on a different GC column (DB-WAX).

The second EAD-active peak had a retention time identical to synthetic 3*Z*,9*Z*-*cis*-6,7-epoxy-21 : H on two columns (DB-5 and DB-WAX). Its mass spectrum exactly matched that of a synthetic standard of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H and data reported in the literature (Millar and Underhill, 1986). In particular, the mass spectrum was characterized by prominent ions at *m/z* 55, 67, 83, 95, and 111, as well as less abundant ions at 223, 277, and 306 (the molecular ion for 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H). The prominent ion at *m/z* 111 is diagnostic for a structure of this type with the epoxide in the 6,7 position (cleavage of C–C bond adjacent to the epoxide; Tóth et al., 1992). However, the enantiomer 3*Z*,9*Z*-6*R*,7*S*-epoxy-21 : H cannot be distinguished from 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H by retention time or mass spectra.

In individual gland extracts of five females analyzed by GC-MS on the DB-5 column, the quantity of 3*Z*,6*Z*,9*Z*-21 : H ranged from 0.12 to 0.51 ng with a mean of 0.23 ± 0.16 ng/gland. The quantity of 3*Z*,9*Z*-*cis*-6,7-epoxy-21 : H ranged from 0.30 to 0.86 ng with a mean of 0.56 ± 0.26 ng. The range in blend ratios of 3*Z*,9*Z*-*cis*-6,7-epoxy-21 : H to 3*Z*,6*Z*,9*Z*-21 : H was 1.59 : 1 to 3.28 : 1 (blend ratio defined as the quantity of the epoxide divided by the quantity of the triene hydrocarbon) with a mean of 2.66 ± 0.68 : 1. Results were similar for independent analyses of five glands conducted on a DB-WAX column. The quantity of 3*Z*,6*Z*,9*Z*-21 : H ranged from 0.19 to 0.65 ng with a mean of 0.36 ± 0.17 ng/gland. The quantity of 3*Z*,9*Z*-*cis*-6,7-epoxy-21 : H ranged from 0.39 to 1.11 ng, with a mean of 0.71 ± 0.32 ng. The range in blend ratios of 3*Z*,9*Z*-

cis-6,7-epoxy-21 : H to 3*Z*,6*Z*,9*Z*-21 : H was 1.31 : 1 to 3.01 : 1, with a mean of 2.12 ± 0.77 : 1.

Field Tests. Following a preliminary field test in which traps baited with 100 μg of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H caught four male *T. mynesalis*, and the enantiomer 3*Z*,9*Z*-6*R*,7*S*-epoxy-21 : H caught no males, we opted to focus the first full field experiment on varying blend ratios of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9*Z*-21 : H. In this experiment, the 1 : 1 blend of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9*Z*-21 : H was the most attractive blend for trapping male *T. mynesalis* (Figure 2). Traps baited with this blend caught significantly more male *T. mynesalis* than control traps [Kruskal-Wallis statistic (H) = 17.73, $P < 0.001$, experimentwise error rate set at $\alpha = 0.05$ for mean comparisons]. The mean numbers of males caught in traps baited with the 4 : 1 and 10 : 1 blends of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9*Z*-21 : H were intermediate between and not significantly different from the numbers caught in control traps (zero) and the 1 : 1 blend, using this conservative statistical test.

In the second field experiment, traps baited with a 1 : 1 blend of 3*Z*,9*Z*,6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9*Z*-21 : H caught the largest number of

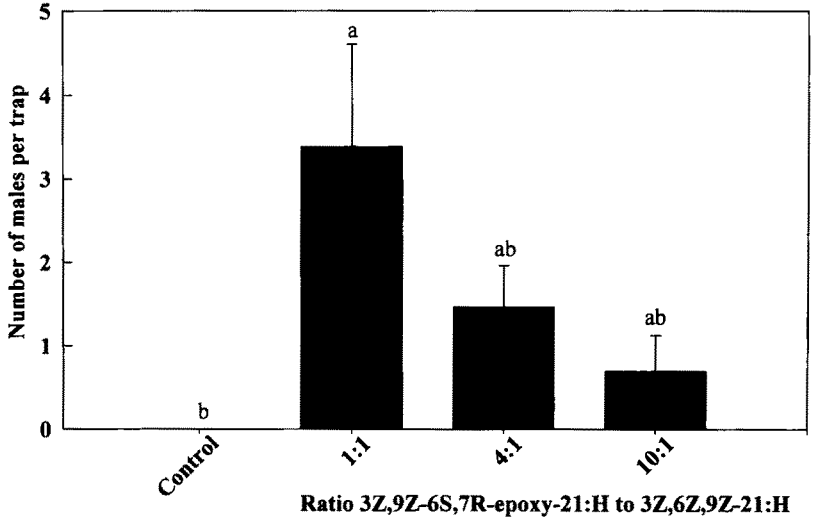

FIG. 2. Captures of male *T. mynesalis* moths in traps baited with blend ratios of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9*Z*-21 : H, or a hexane-treated control. The quantity of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H was kept constant at 100 μg. Bars represent standard errors. Numbers are the mean number of males caught per trap per trapping interval (see Methods and Materials). Means that do not share a letter in common are significantly different (experimentwise error rate $\alpha = 0.05$, Kruskal-Wallis nonparametric analysis of variance).

males and significantly more than all other treatments except for traps baited with 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H alone (Figure 3) ($H = 32.42$, $P < 0.0001$). The enantiomer, 3*Z*,9*Z*-6*R*,7*S*-epoxy-21 : H was ineffective in attracting males to traps either by itself or as part of a 1 : 1 blend with 3*Z*,6*Z*,9*Z*-21 : H. Addition of this enantiomer to the otherwise effective attractant of a 1 : 1 blend of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9*Z*-21 : H shut down the response to the lure.

In the third field experiment, traps baited with 3*Z*,6*Z*,9*Z*-21 : H alone or a 1 : 10 blend of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9*Z*-21 : H were significantly less effective in catching male *T. mynesalis* than traps baited with the 1 : 1 blend ratio (Figure 4) ($H = 45.39$, $P < 0.0001$). The hydrocarbon by itself and the 1 : 10 blend of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9*Z*-21 : H were no more effective than control traps in capturing males in traps.

In the fourth trapping experiment, we focused on a narrower range of blend ratios of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9*Z*-21 : H. The number of males caught in traps baited with blend ratios of 2 : 1, 1 : 1, and 1 : 2 of 3*Z*,9*Z*-6*S*,7*R*-

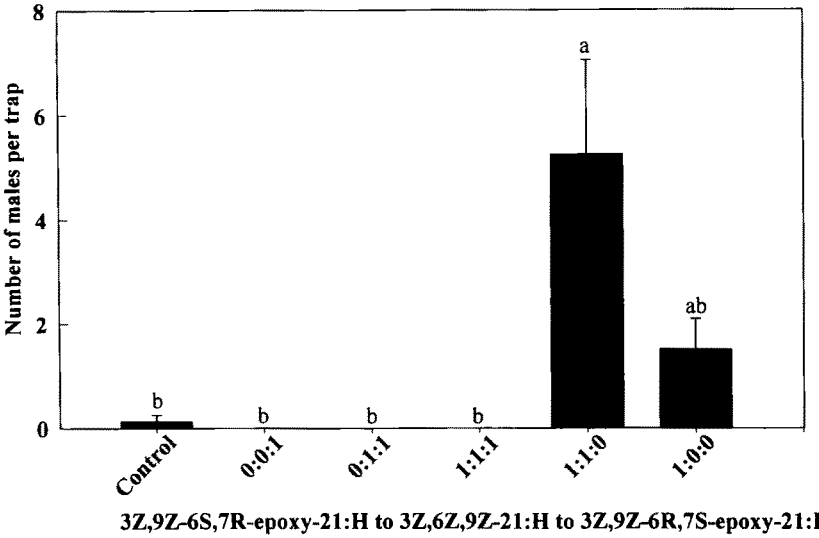

FIG. 3. Captures of male *T. mynesalis* moths in traps baited with blend ratios of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H, 3*Z*,6*Z*,9*Z*-21 : H, and 3*Z*,9*Z*-6*R*,7*S*-epoxy-21 : H, or a hexane-treated control. The quantity of each compound was kept at 100 μg. Bars represent standard errors. Numbers are the mean number of males caught per trap per trapping interval (see Methods and Materials). Means that do not share a letter in common are significantly different (experimentwise error rate $\alpha = 0.05$, Kruskal-Wallis nonparametric analysis of variance).

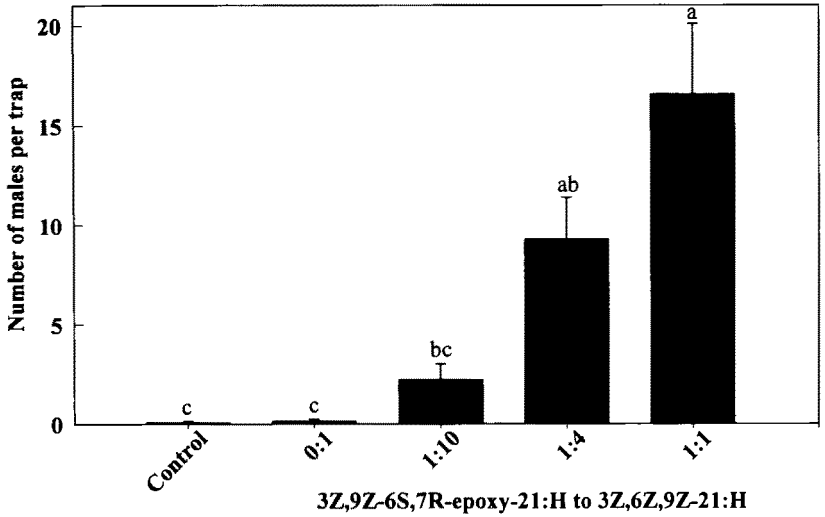

FIG. 4. Captures of male *T. mynesalis* moths in traps baited with blend ratios of 3Z,9Z-6*S*,7*R*-epoxy-21 : H and 3Z,6Z,9Z-21 : H, or a hexane-treated control. The quantity of 3Z,6Z,9Z-21 : H was kept constant at 100 μg. Bars represent standard errors. Numbers are the mean number of males caught per trap per trapping interval (see Methods and Materials). Means that do not share a letter in common are significantly different (experimentwise error rate $\alpha = 0.05$, Kruskal-Wallis nonparametric analysis of variance).

epoxy-21 : H and 3Z,6Z,9Z-21 : H were not significantly different from each other, but each of these blend ratios was significantly more attractive than hexane alone (Figure 5) ($H = 29.59$, $P < 0.0001$).

Blend ratios from 2 : 1 to 1 : 2 of 3Z,9Z-6*S*,7*R*-epoxy-21 : H and 3Z,6Z,9Z-21 : H were effective in capturing male *T. mynesalis* in sticky traps. The range of blend ratios of 3Z,9Z-*cis*-6,7-epoxy-21 : H to 3Z,6Z,9Z-21 : H found in pheromone gland extracts (1.31 : 1 to 3.28 : 1) overlapped this range. The chromatographic techniques we employed did not allow us to distinguish between the enantiomers 3Z,9Z-6*S*,7*R*-epoxy-21 : H and 3Z,9Z-6*R*,7*S*-epoxy-21 : H, and to date, there is no reported analytical chemistry method for distinguishing these two enantiomers at nanogram levels. However, the results from the second field test indicate that 100 μg of 3Z,9Z-6*R*,7*S*-epoxy-21 : H is not effective in attracting males when presented with 100 μg of 3Z,6Z,9Z-21 : H. In addition, the presence of 100 μg of 3Z,6Z,9Z-6*R*,7*S*-epoxy-21 : H within an otherwise effective 1 : 1 blend of 3Z,9Z-6*S*,7*R*-epoxy-21 : H and 3Z,6Z,9Z-21 : H (100 μg each) prevented captures of any males, and thus at this level 3Z,9Z-6*R*,7*S*-epoxy-21 : H is a behavioral antagonist. The enantiomeric purity of the synthetic 3Z,9Z-

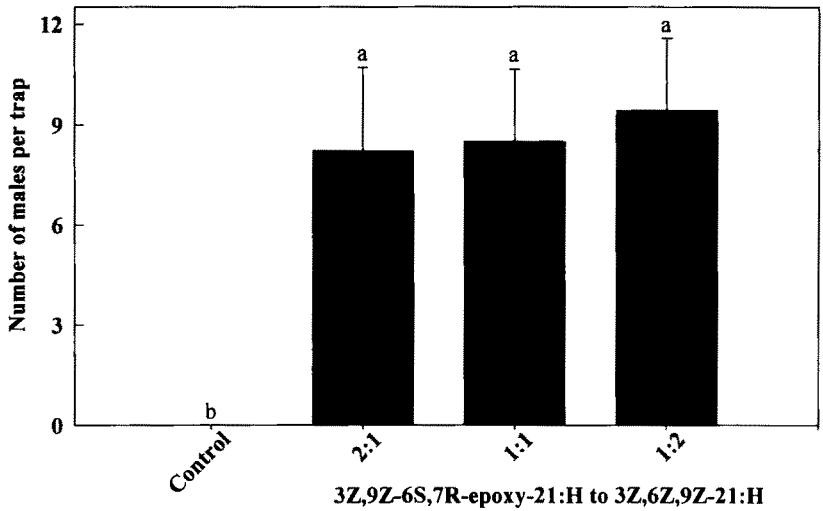

FIG. 5. Captures of male *T. mynesalis* moths in traps baited with blend ratios of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9*Z*-21 : H, or a hexane-treated control. The total quantity of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H plus 3*Z*,6*Z*,9*Z*-21 : H applied to a rubber septum was kept constant at 200 μg. Bars represent standard errors. Numbers are the mean number of males caught per trap per trapping interval (see Methods and Materials). Means that do not share a letter in common are significantly different (experimentwise error rate α = 0.05, Kruskal-Wallis nonparametric analysis of variance).

6*S*,7*R*-epoxy-21 : H was approximately 94% (Millar et al., 1986). This leaves open the possibility that even this 6% level of 3*Z*,9*Z*-6*R*,7*S*-epoxy-21 : H within 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H had a negative (or possibly a positive) impact on captures of males. This cannot be tested until material of higher enantiomeric purity is available.

The identification of a blend of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21 : H and 3*Z*,6*Z*,9*Z*-21 : H as a sex pheromone for *T. mynesalis* is the first such identification for a species in the Herminiinae subfamily of the Noctuidae (Arn et al., 1992). However, in field trials, monoepoxide dienes or trienes (19–21 carbons) have been reported to be sex attractants for other Herminiinae, including *Bleptina caradrinalis* Guenée, *Idia aemula* (Hübner), *I. americalis* (Guenée), and *Palthis angulalis* (Hübner) (Wong et al., 1985; Millar et al., 1991).

Every pheromone blend that has been identified to date for prey species of bolas spiders is composed of unsaturated straight-chain aliphatic aldehydes, alcohols, or acetates (see Yeargan, 1994; Arn et al., 1992). No diene or triene hydrocarbons or their monoepoxide derivatives have been discovered to be sex attractants for any of these prey species. Four noctuid prey species (three species

belonging to Catocalinae and one to Herminiinae) of *M. bisaccata* and two prey species (Geometridae: Larentiinae) of *M. phrynosoma*, however, are closely related to species that have been attracted to specific diene or triene hydrocarbons or monoepoxide derivatives in field screening tests of these compounds. This taxonomic relationship suggests it is very likely that the pheromone blends of a subset of the prey species of these two bolas spiders will be hydrocarbons or epoxides, as is the case for *M. hutchinsoni*.

Prior to the present study, sex attractants or pheromones were known for three of the four species of moths that are captured by *M. hutchinsoni*. Previously, Haynes (1990) determined that female bristly cutworm moths, *Lacinipolia renigera* (Noctuidae: Hadeninae), emit *Z*9–14:Ac and *Z,E*-9,12–14:Ac (3.8% of *Z*9–14:Ac) and that synthetic blends of these two components are attractive to males. Males of the bronzed cutworm, *Nephelodes minians* (Noctuidae: Hadeninae), are attracted to *Z*11–16:Al (Underhill et al., 1977). An optimized attractant for male bluegrass webworms, *Parapediasia teterrella* (Pyralidae: Crambinae), consists of a 20:1 blend of *Z*11–16:Al and *Z*9–16:Al (Clark and Haynes, 1990). Yeargan (1988) found that *T. mynesalis* (52.6%) and *L. renigera* (40.0%) accounted for the vast majority of the 460 moths caught by *M. hutchinsoni* in a two-year study. Thus, the two most frequently captured prey use pheromones that are composed of different classes of chemicals.

The identification of a blend of 3*Z*,9*Z*-6*S*,7*R*-epoxy-21:H and 3*Z*,6*Z*,9*Z*-21:H as a sex pheromone for *T. mynesalis* suggests that female *M. hutchinsoni* may be capable of synthesizing a diverse set of allomones, including both straight-chain aliphatic acetates and aldehydes and triene hydrocarbons and monoepoxide derivatives. Stowe (1988) discussed the evolution of chemical mimicry in plants and animals, including the case of bolas spiders and moths. At the time of his review, no prey species of bolas spiders had been shown to use diene or triene hydrocarbons or their monoepoxide derivatives in their sex pheromones, but the phylogenetic relationships of some prey to species for which these pheromones or attractants had been found suggested that these compounds were likely candidates to be pheromone components of certain prey. Stowe (1988) offered several possible explanations of how bolas spiders might capture moth species that use chemically diverse pheromone components; given the results of our current study, three alternative hypotheses seem most plausible to explain this diversity. First, there may be variation in the attractants produced among individual bolas spiders, resulting in specialization by individual spiders but yielding a more diverse assemblage of prey at the population level. Second, the blend produced by an individual spider might change over time. Last, each spider might produce a consistent single blend of compounds, if individual components do not interfere with attraction of the other species of prey.

Yeargan (1988) found definitive evidence that precludes the possibility of interindividual variation in *M. hutchinsoni* leading to extreme specialization of

individual spiders on a single prey species. He found that the majority of spiders (56.3%) that caught two or more prey items on a single night also caught two or more species of prey. In fact, some individual spiders caught all four of the known prey species over the course of his study.

It is possible that a spider changes the attractant blend it produces over the course of the scotophase, because the two principal prey of *M. hutchinsoni* are captured at different times of night. *L. renigera* is captured before 10:30 PM, while *T. mynesalis* is captured after 11:00 PM, except on very cool nights (Yeargan, 1988). Preliminary evidence suggests that temporal variation may occur in the composition of the blend emitted by *M. cornigera*, the only species of bolas spider for which allomonal components have been identified from the spider itself (Stowe et al., 1987).

An alternative to temporal variation in the allomonal blend produced by *M. hutchinsoni* is that the spider, while hunting, may produce a relatively constant blend of the attractants for the two principal prey species. This would be possible if the chemical constituents do not interfere with the effectiveness of either attractant. The GC-EAD results (Figure 1) indicate that the male *T. mynesalis*'s antenna does not show a measurable EAD response to Z9–14 : Ac (the major pheromone component of *L. renigera*) or Z11–16 : Al (the major sex attractant component for *P. teterrella*). Thus, it is possible that these heterospecific pheromone blends are not even detected by *T. mynesalis*.

Either the second hypothesis (temporal variation) or the third hypothesis (consistent, compatible blend) could account for the ability of *M. hutchinsoni* to capture moths that use chemically diverse pheromones. We plan to investigate this chemical mimicry system to ascertain which hypothesis is correct.

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BIOLOGICAL PERFORMANCE OF COLORADO POTATO BEETLE LARVAE ON POTATO GENOTYPES WITH DIFFERING LEVELS OF POLYPHENOL OXIDASE

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Abstract—Two potato genotypes resistant to the Colorado potato beetle (CPB) and three susceptible genotypes were used to investigate the role of total foliar polyphenol oxidase (PPO) on the performance of CPB larvae in long-term feeding assays. A significant positive correlation was found between larval mortality and PPO content in potato foliage. Moreover, a significant negative correlation was also observed between PPO content and larval weight, fecundity, and relative larval growth rate. These results suggest a significant role of PPO in conferring potato resistance to the Colorado potato beetle at least in those clones where the PPO levels were above a certain threshold.

Key Words—*Leptinotarsa decemlineata*, Colorado potato beetle, polyphenol oxidase, PPO, *Solanum berthaultii*, glandular trichomes, plant resistance.

INTRODUCTION

The high reproductive capacity of the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), its genetic plasticity that allows adaptation to a wide range of environments, and its notorious ability to develop resistance to synthetic

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insecticides (Forgash, 1985) have combined to make this insect the major limiting factor in potato production in the northeastern and Atlantic regions of the United States and a serious problem in Europe (Gauthier et al., 1981; Ferro, 1985). The increasing public concern about environmental problems associated with synthetic insecticides is currently stimulating research directed toward the development of new potato cultivars with resistance to CPB.

Early reports associated the insect resistance of wild potato species with foliar glycoalkaloids (Schreiber, 1957; Pierzchalski and Werner, 1958; and see Tingey 1984 for a review). Most subsequent studies on the response of CPB adults and larvae to specific glycoalkaloids suggest feeding inhibition as the primary resistance mechanism (Sinden et al., 1988).

Another important mechanism of insect resistance in wild potatoes involves the mechanical and chemical properties of their glandular trichomes. Type A and B glandular trichomes of *Solanum berthaultii* Hawkes confer resistance to a number of important insect pests of potato (Gibson, 1971; Tingey and Gibson, 1978; Tingey and Laubengayer, 1986; Tingey, 1991). Most of the work on type A trichomes of *S. berthaultii* has focused on entrapment and immobilization of small arthropods by polymerized trichome exudate (Ryan et al., 1983). Type A trichomes also contain a component that acts as a feeding deterrent to adult CPB (Yencho et al., 1994). The acyl sucroses secreted by type B trichomes enhance entrapment of small arthropods by type A exudate (Gregory et al., 1986); antifeedant activity against aphids and the Colorado potato beetle has also been suggested (Pelletier and Smilowitz, 1990).

Polyphenol oxidase (PPO) is an ubiquitous class of enzymes produced by vascular plants. The type A glandular trichomes of *S. berthaultii* contain a unique PPO with a molecular weight of 59 kDa (Kowalski et al., 1992). Felton et al. (1989) have suggested that PPO-generated quinones might confer post-ingestive insect resistance by reducing the digestibility, palatability, and nutritive value of plant tissue to herbivores. The study reported here was undertaken to indirectly test that hypothesis by comparing performance of CBP larvae on potato clones with differing levels of foliar PPO and differing field resistance to the Colorado potato beetle.

METHODS AND MATERIALS

Plants. Plants of the CPB-susceptible *S. tuberosum* cv. Superior, and the field resistant *S. tuberosum* × *S. berthaultii* hybrid, NYL 235-4 (Plaisted et al., 1992) were grown from disease-free certified seed tubers. Plants of the resistant wild species, *S. berthaultii* (PI 473334), were grown from seeds obtained from the USDA Potato Introduction Station, Sturgeon Bay, Wisconsin. Plants of the

field-susceptible (W.M.T., personal communication) *S. tuberosum* × *S. berthaultii* hybrids N133-67 and N133-93 were grown from nodal cuttings obtained from field-grown plants. All of the genotypes except cv. Superior bear type A glandular trichomes on their foliage. None of these genotypes bear acyl sugar-producing type B glandular trichomes. Plants were grown in 35-cm plastic pots using a peat–perlite soil mix (Pro-Mix B) containing fertilizer (Osmocote 14-14-14 at 2.3 kg/m^3). All plants were grown in a greenhouse at temperatures ranging from 20 to 26°C and relative humidity fluctuating between 90 and 60% in synchrony with temperature. Natural light was supplemented with metal halide illumination of 3600 μE/m^2/sec at plant level to provide a photoperiod of 16 : 8 hr (light–dark). Plants were fertilized weekly with Peters soluble fertilizer (20 : 10 : 20; N-P-K). Plants were about four weeks from emergence or transplanting when used for experimental purposes.

Insects. Eggs of *L. decemlineata* were obtained from a colony reared on *S. tuberosum* cv. Atlantic in greenhouse environmental conditions identical to those described above. This colony is renewed annually with wild adults collected from potato fields in Freeville, New York. Larvae used in experiments came from eggs held in a growth chamber at 26°C and 16 : 8 hr light–dark photoperiod and collected within 24 hr of eclosion.

Foliar glycoalkaloids. Fifty grams of foliage of each clone was collected, frozen, lyophilized, and sent to S.L. Sinden (Vegetable Research Laboratory, USDA, ARS, Beltsville, Maryland) for determination of specific glycoalkaloid content.

Foliar PPO Activity. One leaf immediately below the one to be used for larval confinement was removed and its terminal leaflet was used to estimate total PPO content. Polyclonal antibodies raised against the 59-kDa PPO of *S. berthaultii* trichomes were used to measure PPO content following the method of Kowalski et al. (1992).

Feeding Assays. Larval performance on the selected potato genotypes was determined by confining 10 neonate larvae on the fourth node leaf using a Velcro-sealed 17-cm × 30-cm sleeve cage, made with polyester No See Um insect screening (BaLarval Isom Hercules Corp., Providence, Rhode Island). Six cages were placed on each genotype; plants were held in a greenhouse under the previously described environmental conditions and arranged in a completely randomized block design. Larval mortality and developmental stage were determined at two-day intervals. Larvae were weighed when they began to show borrowing behavior, as evidenced by a change to more yellow color, using a CAHN model DTL 7500 balance. After weight determinations, they were transferred to 500-ml clear plastic cups containing moist soil for pupation. Date of adult emergence was recorded, adults were sexed, and then confined on potato genotypes as described previously. Three pairs of adults were placed in each

cage; cages were replicated three times on each potato genotype. Fecundity was estimated by measuring egg production per female at two-day intervals over an eight-day period following the onset of oviposition. Relative growth rate (RGR) was calculated by ln (wt gain)/days (Felton et al., 1992b).

Amino Acid Analysis of Feces. To evaluate possible antinutritive effects of PPO upon larvae, through chemical degradation of essential amino acids, feces were collected from larvae reared from egg hatch to L4 on each of the potato genotypes as follows: 24 L4 were starved for 9 hr prior to being placed back, as cohorts of six, on leaves at the fourth node of each clone, and confined similarly as above. Thereafter, larvae were fed on each one of those genotypes for 16 hr. Then, all larvae were removed and individually placed into a six-well tissue culture plate (3.5 cm diameter, flat bottom), where they were allowed to defecate for 3 hr after which the feces were immediately frozen at −80°C. Larvae were then transferred to clean plates in the same fashion and allowed to defecate for another 3 hr. One leaflet of each leaf allocated for feeding was removed for determination of its amino acid content using an automated amino acid analyzer at the Cornell Plant Science Center.

Data Analysis. All data were analyzed by one-way analysis of variance (ANOVA). Treatment means were compared by Student-Newman-Keuls or Duncan new multiple-range procedures. Simple regression analysis was made using SUPERANOVA (Abacus Concepts, Inc., Berkeley, California). The P value corresponds to a slope of 0, R is the coefficient of correlation.

RESULTS

Foliar PPO Activity. Foliage of PI 473334 had the highest levels of PPO followed by NYL235-4, N133-67, N133-93, and Superior in decreasing order. Significant differences ($P < 0.05$) were found between PI 473334 and NYL 235-4 and between them and all the other clones. No significant differences were found among the other clones (Table 1).

Foliar Glycoalkaloids. Foliage of the CPB-susceptible cultivar Superior was characterized by the presence of solanine (0.8 mg/g dry weight) and chaconine (1.4 mg/g dry weight) at the highest levels observed for any of the genotypes; no glycoalkaloids were detected in foliage of PI 437334. The same four glycoalkaloids (solamargine, solasonine, solanine, chaconine) were detected in foliage of all *S. tuberosum* × *S. berthaultii* hybrids at levels ranging from 0.5–2.0 mg/g dry weight for solamargine, 0.20–1.23 mg/g dry weight for solasonine, 0.14–0.80 mg/g dry weight for solanine and 0.4–1.4 mg/g dry weight for chaconine. Among the hybrids, levels of solanine and chaconine, two gly-

TABLE 1. LARVAL MORTALITY, FOURTH-INSTAR LARVAL WEIGHT (L4 Wt), AND DAYS TO PUPATION OF *Leptinotarsa decemlineata* FED ON POTATO GENOTYPES WITH DIFFERENT PPO LEVELS

Genotypes	PPO(ABS) Act.[a]	No. Dead larvae[b]	L4 Wt[b]	Days to pupation
Superior	0.240 ± 0.01 a	1.0 ± 0.37 a	186.75 ± 6.0 a	13
N133-93	0.269 ± 0.02 a	1.2 ± 0.48 a	161.25 ± 12.6 a	13
N133-67	0.298 ± 0.02 a	3.3 ± 0.62 b	158.88 ± 12.9 a	13
NYL 235-4	0.944 ± 0.02 b	2.7 ± 0.49 b	172.45 ± 9.1 a	13
PI 473334	1.689 ± 0.06 c	3.7 ± 0.56 b	117.93 ± 9.3 b	15

[a] Values are mean ± SE ($N = 6$). Mean ± SE with the same letter within each category are not significantly different from each other ($P < 0.05$, Newman-Keuls test).

[b] Values are mean ± SE (from an initial $N = 60$; six cohorts of 10). Values with the same letter within each category are not significantly different from each other ($P < 0.05$, Newman-Keuls test).

coalkaloids reported to affect feeding behavior or metabolism of CPB larvae at high concentrations, were particularly low.

Fecal Amino Acid Content. No significant differences were observed in fecal content of the nucleophilic amino acids cysteine, methionine, tryptophan, and lysine from larvae fed on the different potato genotypes (data not shown).

Feeding Assays. Larval mortality was very high (27.0–37.0%) and significantly different from the control ($P < 0.05$) in all clones with high PPO levels, as well as in N133-67, even though the latter clone had PPO contents similar to the control (cv. Superior) and N133-93. A significant positive correlation ($r = 0.49$, $P < 0.01$; $y = 1.43 + 1.36x$) was found between total PPO levels in the potato leaves and CPB larval mortality (Figure 1a). Conversely, larval weight was similar for all clones regardless of the PPO levels except for *S. berthaultii* PI 473334, where larval weight was significantly ($P < 0.05$) reduced. However, a highly significant negative correlation ($r = -0.53$, $P < 0.01$; $y = 180.4 - 30.4x$) was observed between larval weight and PPO (Figure 1b). Likewise, the larval development (number of days to pupation) was the same for all cases except PI 473334, where a two-day delay was observed (Table 1).

The number of eggs per female was significantly reduced ($P < 0.05$) on all clones with high PPO levels relative to cv. Superior. Although there were no differences in the number of egg masses per female, the number of eggs per mass was significantly different between PI 473334 and the clones with low

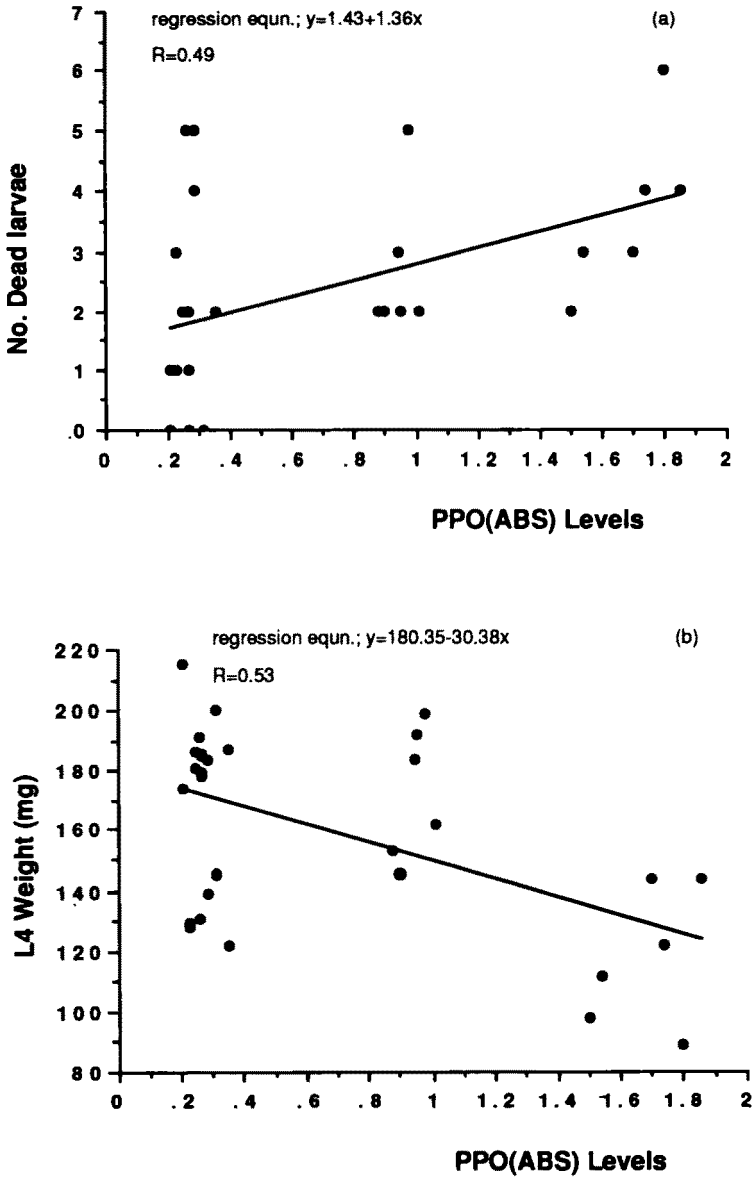

FIG. 1. Relationship between the PPO levels and (a) larval mortality and (b) the fourth-instar larval weights.

TABLE 2. EGGS PER FEMALE, EGG MASSES PER FEMALE, AND EGGS PER MASS OF *Leptinotarsa decemlineata* FED ON POTATO GENOTYPES WITH DIFFERENT PPO LEVELS

Genotypes	PPO(ABS) Act.[a]	Eggs/female[b]	Egg masses/ female[b]	Egg/mass[b]
Superior	0.240 ± 0.01 a	727.14 ± 45.7 b	15.540 ± 0.8 a	41.266 ± 1.8 b
N133-93	0.269 ± 0.02 a	651.93 ± 13.5 ab	13.667 ± 0.4 a	40.633 ± 0.4 b
N133-67	0.298 ± 0.02 a			
NYL 235-4	0.944 ± 0.02 b	611.78 ± 12.2 a	14.150 ± 0.2 a	37.530 ± 2.7 ab
PI 473334	1.689 ± 0.06 c	535.13 ± 31.9 a	15.233 ± 0.4 a	33.123 ± 2.0 a

[a] Values are mean ± SE ($N = 6$). Mean ± SE with the same letter within each category are not significantly different from each other ($P < 0.05$, Newman-Keuls test).

[b] Values are mean ± SE ($N = 5$ replicates of four couples for Superior, $N = 4$ for NYL 235-4, and $N = 3$ for N133-93 and PI473334. Values for N133-67 are missing due to less than three replicates available). Mean ± SE with the same letter within each category are not significantly different from each other ($P < 0.05$, Duncan's new multiple-range test).

PPO activity (Table 2). A significant negative correlation ($r = 0.57$, $P < 0.01$; $y = 677.7 - 82.2x$) was found between the number of eggs per female and PPO activity (Figure 2a). Total PPO in foliage was strongly negatively correlated ($r = 0.8$, $P < 0.001$; $y = 0.45 - 0.05x$) with relative growth rate (RGR) of larvae (Figure 2b). No differences were found in the sex ratio regardless of PPO contents among clones.

DISCUSSION

Our study on prolonged ingestion of potato foliage by CPB (from neonate to pupation), suggests that PPO has a negative effect on CPB. We have shown a differential effect of several potato genotypes on the performance of Colorado potato beetle. Several potato allelochemicals, such as the leptine glycoalkaloids, have been implicated in resistance to this beetle (Sinden et al., 1991). However, other glycoalkaloids, e.g., solasonine and solamargine have no effect on its feeding behavior or metabolism, at biologically relevant levels (Gregory et al., 1981; Tingey and Sinden, 1982). Furthermore, these two glycoalkaloids also occur in *S. rostratum*, one of the Colorado potato beetles' original wild hosts (Schreiber, 1968). These facts along with the low concentrations of solasonine and solamargine in our plants suggests that these compounds did not play a role in the results obtained here.

For *S. berthaultii* (highest PPO levels), larval mortality was very high (37.0%), larval development was extended two days, and there was a significant

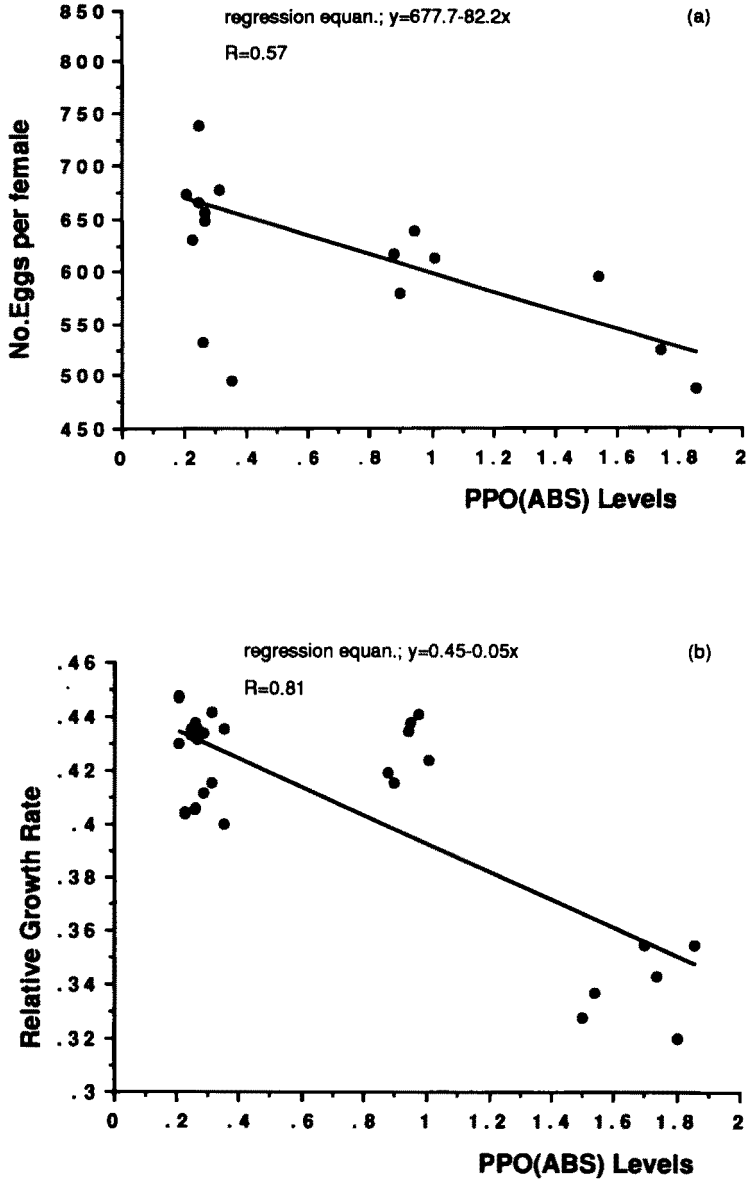

FIG. 2. Relationship between the PPO levels and (a) fecundity and (b) the relative larval growth rate of CPB.

reduction ($P < 0.05$) in late fourth-instar (L4) weight when compared to cv. Superior (Table 1). Not all of these effects are likely to be associated strictly with PPO content. For example, N133-67 has about the same PPO level as that of Superior and N133-93, but larval mortality was in the range of the genotypes with three-fold and five-fold greater PPO levels. However, the data strongly suggest an association between total PPO levels and CPB larval performance, statistically supported by a significant positive correlation between PPO and larval mortality (Figure 1a). Moreover, a negative correlation was found between foliar PPO levels and L4 weight, fecundity, and the relative growth rate (Figures 1b and 2). These data suggest that PPO plays a role in resistance to CPB larvae, at least in those clones where the PPO content is above a certain threshold.

Recently, Felton et al. (1992a) reported that polyphenol oxidases are unlikely to have significant antinutritive effects on the Colorado potato beetle. However, their data were based on a short-term feeding experiment using fourth-instar CPB larvae, and total foliar PPO was not considered. In contrast, our data were obtained from a much longer feeding study, possibly leading to a cumulative effect. In fact, nearly 75% of the larvae died during the second and third instars on those clones with high PPO activity. Similarly, field resistance of NYL 235-4 is expressed by high mortality during the first and second instars (Plaisted et al., 1992).

Lowest mortality (about 9%) was observed during the fourth instar. This may partially explain why we did not find significant differences in the essential nucleophilic amino acids cysteine, methionine, tryptophan, and lysine in the feces of late fourth-instar CPB larvae. This result is in agreement with Felton et al. (1992a), who suggested that the mildly acidic midgut of CPB larvae would impede the covalent conjugation of amino acids with the electrophilic quinones, thus preventing the loss of essential amino acids. Nevertheless, the high mortality observed in the second and third-instar CPB suggests that other physiological and/or biochemical properties of the PPO–digestive system are implicated. In fact, PPO has the potential to confer resistance because its end products, O-quinones, might alkylate foliar protein, leading to reduced digestibility and nutritive value, as reported for other insect species (Felton et al., 1989; Duffey and Felton, 1991). Glandular trichome PPO (foliar PPO in our conditions) is one component of an interacting system of several chemical and physical characteristics that result in the expression of insect resistance (Kowalski et al., 1990). This work is a first step in unraveling the complex of biochemical mechanisms that underlay the ecological interactions between trichome A-mediated resistance of *S. berthaultii* and the Colorado potato beetle. Future studies need to address the effects of PPO on feeding and physiology of CPB, particularly now that transgenic plants with different levels of PPO can be obtained.

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SEX PHEROMONE OF CADDISFLY *Hesperophylax occidentalis* (BANKS) (TRICHOPTERA: LIMNEPHILIDAE)

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Abstract—The main component of the sex pheromone of the caddisfly *Hesperophylax occidentalis* (Banks) (Trichoptera: Limnephilidae) was identified as 6-methylnonan-3-one (enantiomeric composition has not yet been determined). Extracts of adult females elicited strong electroantennogram (EAG) responses from adult male antennae, but elicited significantly smaller EAG responses from adult female antennae. Extracts of adult males did not elicit appreciable EAG responses from antennae of either sex. Racemic 6-methylnonan-3-one was synthesized and elicited EAG responses from male antennae as strong as those obtained with female extracts. In field tests with baited sticky traps near lakes and streams, traps baited with synthetic racemic 6-methylnonan-3-one caught significantly more males than control traps. Female adults contained approximately 1 μg of 6-methylnonan-3-one per individual. Related ketones and alcohols of other chain lengths were also tentatively identified, being present in tiny amounts in female extracts. Extraction of different body parts showed that 6-methylnonan-3-one occurs only in a region near the intersegmental membrane between the fourth and fifth abdominal sternites of the female (no discrete glands were observed). Extracts of males did not contain 6-methylnonan-3-one, nor did pupae of either sex.

Key Words—Electroantennogram, EAG, caddisfly, Trichoptera, Limnephilidae, pheromone, attractant, semiochemical, infochemical, mating, sex, volatiles.

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INTRODUCTION

Scent production in caddisflies was first discussed by naturalists near the end of the last century (Müller, 1888; Cummings, 1914; Elteringham, 1919; Mosely, 1919, 1922, 1923), and it was suggested that these chemical secretions and similar ones from Microlepidoptera may serve a role in defense against predation (Kimmins, 1956; Kristensen, 1972). The possible role of volatile compounds in mating communication in the Trichoptera was not considered until relatively recently (Kelner-Pillault, 1975; Wood and Resh, 1984; Resh and Wood, 1985; Resh et al., 1987), despite the fact that sex pheromones have been characterized for more than a thousand insect species (Mayer and McLaughlin, 1991), distributed among nine orders (Orthoptera, Homoptera, Hemiptera, Lepidoptera, Coleoptera, Mecoptera, Neuroptera, Hymenoptera, and Diptera). The majority of the sex pheromones so far identified are for species in the Lepidoptera (Mayer and McLaughlin, 1991). The first chemical identifications of sex pheromones in the Trichoptera (Hydropsychidae and Rhyacophilidae) were reported only recently (Valeur et al., 1990; Löfstedt et al., 1994).

The first report of sex pheromone communication in the Trichoptera was for the sericostomatid *Gumaga griseola* (McLachlan) and *Gumaga nigricula* (McLachlan) (Wood and Resh, 1984). Field experiments showed that males are attracted to caged females and to female extracts (Wood and Resh, 1984; Resh et al., 1987). A female-produced sex pheromone has been found in *Rhyacophila nubila* (Zetterstedt) (Rhyacophilidae), and males were attracted to sticky traps baited with females only during the early hours of the night (Solem, 1985). Other researchers have also demonstrated sex pheromone communication in caddisflies, including the Glossosomatidae (Resh et al., 1987), Limnephilidae (Kelner-Pillault, 1975; Resh and Wood, 1985), Philopotamidae (Wood and Resh, 1984), Helicopsychidae (Resh et al., 1984), and Molannidae (Solem and Petersson, 1987), by conducting field tests with live females or with extracts from different female parts. Field trapping experiments with extracts of specific body sections from adult female *Gumaga nigricula* (Sericostomatidae), *Gumaga griseola* (Sericostomatidae), and *Dicosmoecus gilvipes* (Hagen) (Limnephilidae) have implicated the fifth abdominal sternite in pheromone release (Resh and Wood, 1985). Mating communication in *Mystacides azurea* L. (Leptoceridae) and *Athripsodes cinereus* Curtis (Leptoceridae) apparently does not involve sex pheromones, and visual cues seem to be more important (Petersson and Solem, 1987).

Hesperophylax occidentalis (Banks) is a univoltine species common in temporary streams at high elevations or latitudes (Anderson, 1976). Eggs are laid in a single mass just below the water's surface (Parker and Wiggins, 1985), and adults emerge from April to October, typically with peaks in May and June. The life history and ecology of *Hesperophylax occidentalis* have been described

(Martinson and Ward 1982). The present study on *Hesperophylax occidentalis* is the first chemical identification of a sex pheromone for a species in the family Limnephilidae.

METHODS AND MATERIALS

Insects. Pupae were found attached by silk strands to the undersides of rocks. They were collected at two locations. In Colorado, pupae were collected from late May to late July from the upper streambed of Hewlett Gulch in Roosevelt National Forest (approximately 20 miles west of Fort Collins, Colorado). In Wyoming, pupae were collected from the edge of Glacier Lakes in Medicine Bow National Forest throughout August. Pupae were placed in an insulated plastic picnic cooler (4-liter capacity, Rubbermaid, Gott Corp., Winfield, Kansas) and covered with water from the stream or lake from which they were collected. A battery-powered air pump (ordinarily used to keep fishing bait alive; B&M Air Pump, West Point, Mississippi 39773) provides constant aeration to the pupae through an airstone. Adults often began emerging from the pupae within an hour after collection, and emergence continued for several weeks after return to the laboratory. Continuous aeration of pupae with an aquarium pump was maintained after return to the laboratory from a field site. Twigs were placed at one end of the container (protruding from the water) to allow adults an emergence site.

For one experiment (comparison of male and female electroantennogram responses to extracts of both sexes), adults were collected from Endovalley Campground at Rocky Mountain National Park, Colorado, in September, under the auspices of a permit issued to P.A. Opler. Adults were attracted to a white sheet (2 × 2 m) laid on the ground, with a mercury vapor lamp (140 W, Electripak, Box 30489, Memphis, Tennessee 38130-0489) in the center, powered by a gasoline-powered generator (Honda model EX1000). Adults were placed in Ziploc freezer bags (4-liter size, DowBrands L.P., Indianapolis, Indiana) and stored in a refrigerator (5°C).

Extracts. Extracts were made by placing different body parts of males and females in 1 ml of dichloromethane in a 4-ml glass vial with a Teflon-lined cap for 1 hr. Body parts were separated by using fine forceps to tear between tergites. Preliminary experiments demonstrated that male antennae respond electrophysiologically to extracts of female abdomens and that ventrum extracts were more active than tergum extracts.

The site of pheromone storage was determined by cutting the ventrum into pieces with a razor blade and extracting each piece separately with dichloromethane. Cuts were made transversely through the center of each sternite, so as to include the intersegmental membrane between successive sternites.

Gas–Liquid Chromatography. To obtain purified material for identification, a dichloromethane extract of 25 female ventra was separated into fractions with a packed OV-101 column (3 mm × 3 m, glass) with temperature programming from 60°C to 250°C at 10°C/min (1 min delay) on a Hewlett-Packard 5890 gas–liquid chromatograph (GLC). Each fraction was collected for 1 min in a 20-cm Pasteur pipet (10 cm of this was a glass capillary), with the tip of the pipet inserted into a Teflon fitting attached to the jet at the end of the GLC. Compounds were recovered from the pipet by attaching a rubber bulb to the pipet and drawing distilled dichloromethane up and down in the capillary portion of the pipet several times. Capillary gas–liquid chromatography with flame ionization detection was conducted with two columns, a nonpolar RSL-150 column (0.25 mm × 25 m, Applied Science) and a polar Supelcowax column (0.25 mm × 25 m, Applied Science).

Mass Spectrometry. Electron ionization–mass spectrometry (GC-EI-MS) was conducted with a HP 5790 mass spectrometer interfaced with a HP 5890 gas chromatograph, using a methyl silicone capillary column in splitless mode (0.25 mm × 25 m, Applied Science) with temperature programming from 60°C to 250°C at 10°C/min (1 min delay). Chemical ionization–mass spectrometry (GC-CI-MS) was conducted similarly, with ammonia as the reagent gas. A computer data base of electron ionization mass spectra (approximately 15,000 compounds) from the National Bureau of Standards was available on the HP 5790 mass spectrometer.

Lithium Aluminum Hydride Reaction. Preliminary evidence from EI-MS indicated that the main pheromone component was a 3-ketone. A larger amount of this main pheromone component was purified by GLC and then derivatized with lithium aluminum hydride (LAH) to prepare the corresponding alcohol for further characterization. The purified main component from GLC collection was placed in 0.5 ml diethyl ether in a 4-ml glass vial with a Teflon-lined cap, and 20 mg LAH was added with a microspatula (Fieser and Fieser, 1960). For destruction of excess LAH, 3 ml of ether was shaken with water in a separate vial, and this "wet" ether solution was added dropwise to the LAH reaction of the main pheromone component. The small amount of water in the "wet" ether destroyed excess LAH with a minimum of fizzing. When no further reaction was observed upon dropwise addition of "wet" ether, the ether solution was washed three times with water and filtered through a small piece of a Kimwipe (disposable cellulose wiper, Kimberly-Clark Corp., Neenah, Wisconsin 54956) in a glass Pasteur pipet. The solvent was removed by evaporation with a nitrogen stream, and 0.5 ml of dichloromethane was added to redissolve the product. The product was analyzed by GC-EI-MS.

Triphenylphosphine Dibromide Reaction. The alkyl bromide analog of the main pheromone component was prepared with triphenylphosphine dibromide (Wiley et al., 1964). Triphenylphosphine dibromide was prepared by adding

bromine dropwise with shaking to a solution of triphenylphosphine (50 mg) in dichloromethane (1 ml) until a yellow color persisted, then adding more triphenylphosphine until the solution lost the yellow tint. The LAH product of the main pheromone component (in dichloromethane solution) was added to the triphenylphosphine dibromide solution and shaken gently for 1 min. Evaporation with a nitrogen stream was used to remove nearly all the solvent, and hexane (1 ml) was added to precipitate unreacted triphenylphosphine dibromide. The solution was filtered through a small piece of a Kimwipe in a glass Pasteur pipet, solvent was removed by evaporation with a nitrogen stream, and hexane (1 ml) was added. This solution was applied to the top of a small chromatograph column made from a Pasteur pipet half-filled with a slurry of Florisil in hexane. Elution of the alkyl bromide was carried out with 4 ml of hexane, leaving behind any unreacted alcohol. The product was analyzed by GC-EI-MS.

Sodium Borohydride Reaction. The hydrocarbon skeleton was prepared from the alkyl bromide derivative of the main pheromone component by using sodium borohydride in dimethyl sulfoxide (DMSO), as described by Hutchins et al. (1969). Solvent was removed from a solution of the alkyl bromide derivative of the main pheromone component by evaporation with a nitrogen stream, and 0.5 ml of DMSO was added. Sodium borohydride (20 mg) was added with a microspatula, the vial was capped (with a Teflon-lined cap), and the vial was placed in an oven (85°C) for 18 hr. The vial was cooled, hexane (1 ml) was added, and the hexane solution was washed three times with water. The hydrocarbon skeleton was analyzed by GC-EI-MS.

Synthesis of Main Pheromone Component. Synthesis of racemic 6-methylnonan-3-one was carried out in three steps. Racemic 4-methylheptan-1-ol (Wiley Organics, Coshocton, Ohio) was oxidized by constant stirring (24 hr) with pyridinium dichromate (PDC, Aldrich Chem. Co., Milwaukee, Wisconsin) in dichloromethane to form 4-methylheptanal (Corey and Schmidt, 1979). Particulate material was removed by centrifugation after the reaction was complete. The aldehyde was purified by elution through a column of Florisil with a 1 : 1 mixture of dichloromethane and diethyl ether. Racemic 6-methylnonan-3-ol was prepared by adding ethylmagnesium bromide (3 *M* solution in diethyl ether; Aldrich Chem. Co.) to 4-methyl-heptanal under nitrogen (Fieser and Fieser, 1967). Unreacted ethylmagnesium bromide was destroyed by adding a water-saturated solution of diethyl ether, and particulate material was removed by centrifugation. Racemic 6-methylnonan-3-one was prepared from 6-methylnonan-3-ol by constant stirring (24 hr) with PDC in dichloromethane (Corey and Schmidt, 1979) and was purified by elution from Florisil with a 1 : 1 mixture of dichloromethane and diethyl ether.

Electroantennograms. Electroantennogram (EAG) tests were conducted as described by Bjostad (1988). An antenna was plucked from the head with fine forceps, cut at the distal end, and suspended between the tips of the electrodes,

made from glass Pasteur pipet containing 0.15 M KCl saline solution with Ag–AgCl wires in contact with the saline solution. The signal from the antenna was amplified (5000×, FET amplifier) and processed with an analog-to-digital converter for recording with a microcomputer. Antennae used for EAG tests were taken from insects within a few days of adult emergence (typically two to three days).

Field Trapping. Trécé 1C wing traps (Pest Management Supply, Inc., Hadley, Massachusetts) were used to test synthetic pheromone lures. This trap type has previously been used to trap Trichoptera (Resh and Wood, 1985; Resh et al., 1987). Lures were prepared by placing dichloromethane solutions of racemic 6-methylnonan-3-one in the large ends of rubber serum stoppers (Aldrich Chem. Co.) and allowing the solvent to evaporate in a chemical fume hood.

Traps were tested along the lakesides of Glacier Lakes in Medicine Bow National Forest, Wyoming, in July, August, and September (*H. occidentalis* pupae were found in abundance just offshore). A lure was placed in the center of each trap, and the trap was placed in a tree approximately 1 m above the ground. Sex pheromone release takes place at low light intensity in other caddisfly species (Benz, 1975; Solem, 1985; Resh et al., 1987), and traps were accordingly set out a few hours before dark. It has been previously demonstrated with other caddisfly species that lures are attractive for several days (Resh et al., 1987), and lures were replaced every three to seven days in the present study. Placement of traps can have a substantial influence on the relative numbers of adults captured (Cardé and Elkinton, 1984). Males of other caddisfly species have been observed to patrol bushes along the banks of rivers, presumably in search of females (Solem, 1984, 1985). Traps for *H. occidentalis* were placed about 10 m apart in vegetation on the banks of both the lake and stream approximately 1 m from the ground, similar to methods used by Resh et al. (1987).

Adult caddisflies were removed from traps, and sex was determined by examining the genitalia (Parker and Wiggins, 1985). Species and sex of specimens were determined by Dr. Boris Kondratieff (Colorado State University, Fort Collins, Colorado) and by David Ruiter (Environmental Protection Agency, Denver, Colorado). Female abdomens were brown and had a noticeable odor (to the human nose), and abdomens of males were pale green and odorless.

Statistical Analyses. Data were analyzed by one-way analyses of variance. Differences among means were determined by Newman-Keuls multiple-range test (Winer, 1971).

RESULTS

In EAG tests, male antennae responded strongly to extracts of female abdomens, and female antennae responded significantly less (Figure 1). Extracts of

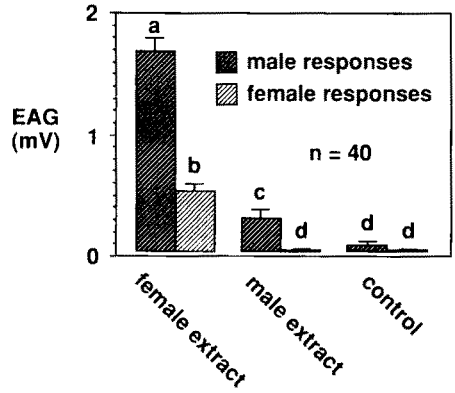

FIG. 1. Male and female responses to male and female extracts; n = total number of responses to each treatment recorded from eight males and 10 females. Bars on each column indicate standard error. Treatments denoted by the same letter are not significantly different with Newman-Keuls multiple-range test ($P = 0.05$).

male abdomens produced weak EAG activity with both male and female antennae. Males and females for this experiment were collected from a mercury vapor lamp and carried to the laboratory in Ziploc bags.

Capillary GLC-FID of a female abdominal extract on a methyl silicone column showed one major peak and a number of smaller peaks (Figure 2). A single area of EAG activity from male antennae was observed among gas chromatographic fractions (methyl silicone, packed column) that were collected from

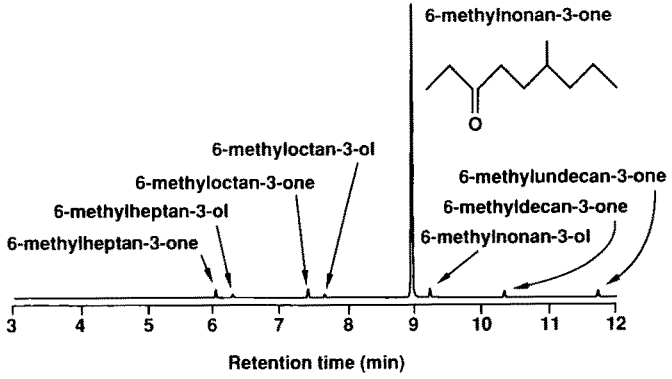

FIG. 2. Capillary gas–liquid chromatogram (methyl silicone) of *Hesperophylax occidentalis* female abdominal extract.

a female extract. The most EAG-active fraction (6–8 min) contained essentially all of the major peak observed previously by capillary GLC (Figure 2). EAG activity in subsequent fractions was apparently due to slight tailing of this compound (above the EAG threshold of the male antennae).

EI-GC-MS analysis of the major peak in female extracts indicated an apparent mass of 156 (Figure 3A). Probability-based matching with a National Bureau of Standards computer data base of mass spectra suggested that the compound was an alkan-3-one. The retention time of this major peak was 7.05 min, shorter than the retention time of synthetic decan-3-one (7.63 min) and longer than the retention time of synthetic nonan-3-one (5.78 min). This suggested that the compound was a methyl-branched nonan-3-one. CI-GC-MS analysis (with ammonia as the reagent gas) of the major peak in female extracts indicated a base peak of 174 (M+18), corroborating the mass of the compound as 156 (Figure 3B).

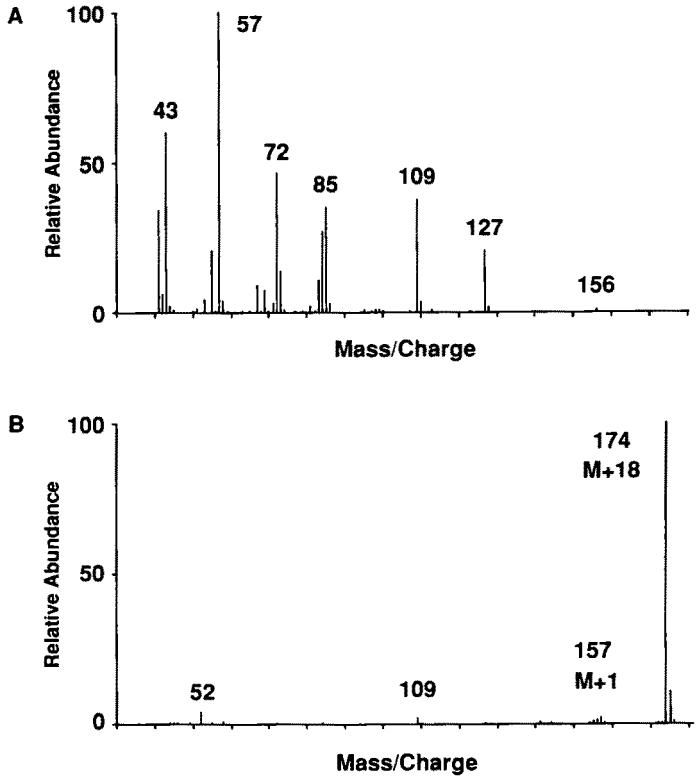

FIG. 3. (A) Electron ionization mass spectrum and (B) chemical ionization mass spectrum of 6-methylnonan-3-one extracted from a female *Hesperophylax occidentalis*.

The hydrocarbon skeleton was prepared from the main pheromone component in order to determine the position of the methyl branch. The mass spectra of all monomethyl nonanes are in the literature and differ appreciably from one another (Stenhagen et al., 1974). Derivatization of the main pheromone component with LAH produced a compound with a slightly later retention time (7.20 min). EI-GC-MS analysis of this compound indicated a mass of 158 (consistent with a monomethyl-nonanol), and probability-based matching with a National Bureau of Standards mass spectral data base suggested that the compound was an alkan-3-ol. Derivatization of this alcohol with triphenylphosphine dibromide produced a compound with a later retention time (8.49 min), and EI-MS of this compound was consistent with a monomethyl bromononane. The hydrocarbon skeleton (retention time 4.22 min) was prepared from the monomethyl bromononane with sodium borohydride in DMSO, and EI-MS indicated that this compound was 4-methyl-nonane, with diagnostic peaks at *m/z* 142 (M+, 4%), 57 (100%), 43 (80%), 41 (44%), 70 (38%), 71 (36%), 98 (26%), and 99 (10%). Nonanes with methyl branches at other positions have substantially different mass spectra (Stenhagen et al., 1974).

Two structural possibilities existed at this point. The derivative 4-methylnonane would be generated as a hydrocarbon skeleton from either 6-methylnonan-3-one or 4-methylnonan-3-one. To distinguish between these possibilities, the fragments in the EI mass spectrum of the parent pheromone component were considered in detail (Figure 4). The parent pheromone component was initially identified as 6-methylnonan-3-one on the basis of diagnostic fragments at *m/z* 72 and *m/z* 84 (4-methylnonan-3-one would be expected to have diagnostic fragments at *m/z* 70 and *m/z* 86). Synthetic 6-methylnonan-3-one (racemic) prepared in our laboratory had a retention time (7.05 min) and EI mass spectrum that were identical to those for the isolated main pheromone component from females. GC-EI-MS of related compounds was conducted for comparison, including 7-methylnonan-3-one (retention time 7.07 min) and 2-methylnonan-3-one (retention time 6.82 min), and both compounds had mass spectra easily distinguishable from that of 6-methylnonan-3-one.

Gas chromatographic analysis of extracts of separate pieces of the female ventrum indicated that 6-methylnonan-3-one occurs selectively in the region between the midpoints of the fourth and fifth abdominal sternites (the pieces were obtained by cutting transversely through the midpoint of each sternite, so as to include the intersegmental membrane between successive sternites). No discrete glands were observed, however. Approximately 1 μg of 6-methylnonan-3-one was found per female. Other compounds were also found in small amounts in female extracts, and these were tentatively identified on the basis of mass spectra and relative retention times as 6-methyl-alkan-3-ones, including 6-methylheptan-3-one [retention time 3.61 min, with diagnostic peaks at *m/z* 99 (8%), 86 (50%), 71 (75%), 57 (100%), and 43 (78%)], 6-methyloctan-3-one [retention time 5.56 min, with diagnostic peaks at *m/z* 113 (20%), 95 (49%), 85 (18%),

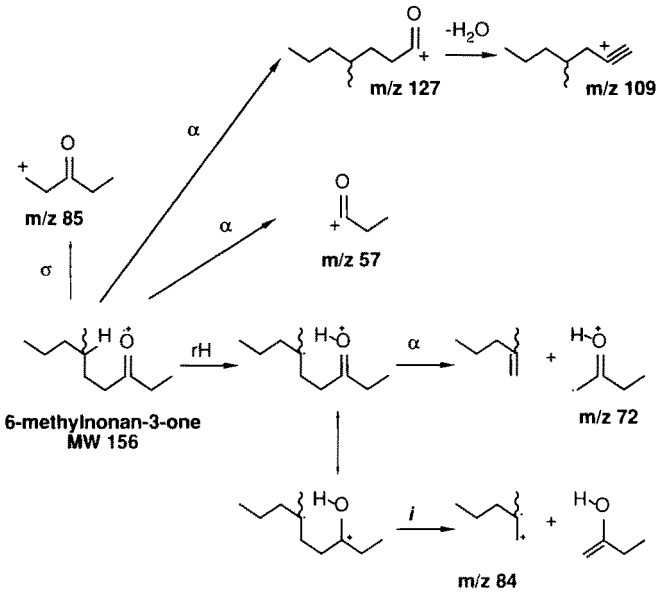

FIG. 4. Proposed fragmentation pattern for electron-ionization mass spectrum of 6-methylnonan-3-one.

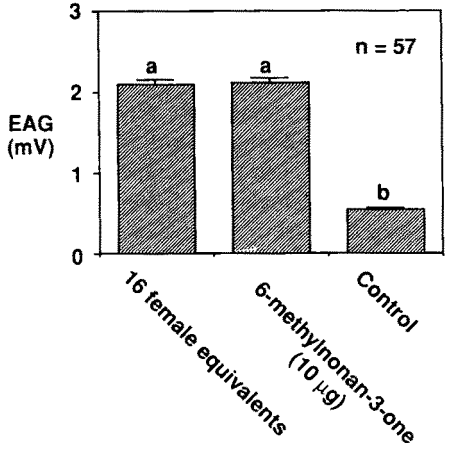

FIG. 5. Male *Hesperophylax occidentalis* EAG responses; n = total number of recorded responses to each treatment. Bars on each column indicate standard error. Treatments denoted by the same letter are not significantly different by Newman-Keuls multiple-range test ($P = 0.05$).

72 (48%), 57 (100%), and 43 (77%)], 6-methyldecan-3-one [retention time 8.50 min, with diagnostic peaks at *m/z* 141 (17%), 123 (6%), 98 (20%), 85 (24%), 72 (48%), 57 (100%), and 43 (52%)], and 6-methylundecan-3-one [retention time 9.95 min, with diagnostic peaks at *m/z* 155 (14%), 137 (7%), 112 (18%), 72 (49%), 57 (100%), and 43 (63%)]. Small amounts of the corresponding alcohols were also tentatively identified for some of these compounds, including 6-methylheptan-3-ol [retention time 4.25 min, with diagnostic peaks at *m/z* 101 (9%), 83 (16%), 59 (100%), and 41 (38%)], 6-methyloctan-3-ol [retention time 5.85 min, with diagnostic peaks at *m/z* 115 (22%), 97 (74%), 69 (39%), 59 (64%), 55 (100%), and 41 (60%)], and 6-methylnonan-3-ol [retention time 7.20 min, with diagnostic peaks at *m/z* 129 (23%), 111 (29%), 69 (100%), 59 (96%), 55 (43%), and 41 (53%)]. A small amount of indole [retention time 11.95 min, with diagnostic peaks at *m/z* 151 (100%), 124 (13%), 116 (23%), and 89 (41%)] was observed in some extracts, but not in others.

To verify that the main pheromone component is 6-methylnonan-3-one, EAG responses of male antennae to synthetic 6-methylnonan-3-one (racemic) and to extracts of female abdomens containing a comparable amount of the main pheromone component were compared (Figure 5). Responses of male antennae were equal for 16 female equivalents and for 10 μg of 6-methylnonan-3-one (racemic).

Traps baited with racemic 6-methylnonan-3-one caught almost exclusively male *H. occidentalis*, and the males were selectively attracted to the 100-μg lures, with higher and lower amounts attracting significantly fewer males (Figure 6A). A small number of females were also trapped, but the numbers of females caught in traps baited with 6-methylnonan-3-one were not significantly different from the number caught in control traps (Figure 6B).

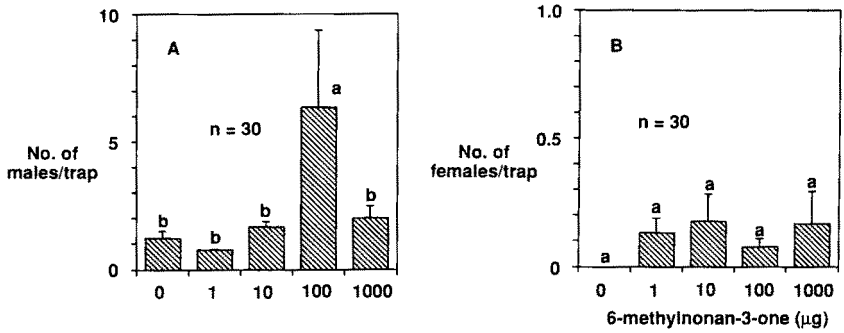

FIG. 6. (A) Males and (B) females caught in traps baited with different amounts of synthetic, racemic 6-methylnonan-3-one; n = number of times adults were counted. Bars on each column indicate standard error. Treatments denoted by the same letter are not significantly different by Newman-Keuls multiple-range test (P = 0.05).

DISCUSSION

The main sex pheromone component of *Hesperophylax occidentalis* is 6-methylnonan-3-one. This is apparently the first report of this compound as a natural product. We have not yet determined the enantiomeric composition of the 6-methylnonan-3-one from female *Hesperophylax occidentalis* (this presumably will be quite difficult to determine for a methyl group with no other functional groups nearby). Smaller amounts of several structurally related compounds were also tentatively identified in extracts of pheromone glands, including 6-methylheptan-3-one, 6-methyloctan-3-one, 6-methyldecan-3-one, 6-methylundecan-3-one, 6-methylheptan-3-ol, 6-methyloctan-3-ol, and 6-methylnonan-3-ol. Synthetic 6-methylnonan-3-one (racemic) prepared in our laboratory was active in EAG tests and also in field trapping tests with male *H. occidentalis*. Further EAG tests with analogs of 6-methylnonan-3-one have been conducted, including chain-length analogs, keto-position analogs, and methyl-group-position analogs (Jewett, 1993). Small EAG responses from male antennae were observed in response to male abdomen extract (Figure 1), but this may simply be due to slight contamination of the extracted males with volatile compounds from females during transport from the field to the laboratory.

Males were selectively caught in traps containing 100 μg of synthetic 6-methylnonan-3-one. Small numbers of females were also trapped, but the numbers of females caught in traps baited with different doses of 6-methylnonan-3-one were not significantly different from one another nor from the number caught in control traps (Figure 6B). This implies that males were indeed attracted to the synthetic pheromone, not to the small numbers of females that were incidentally caught in some of the traps (in fact, qualitatively it was observed that the smallest number of females was caught in traps containing the 100-μg lures).

The first chemical identifications of pheromones shown formally to be involved in sexual communication have recently been reported (Valeur et al., 1990; Löfstedt et al., 1994) for two caddisfly species, *Rhyacophila fasciata* (Hagen) (Rhyacophilidae) and *Hydropsyche angustipennis* (Curtis) (Hydropsychidae), and possible pheromone components (no behavioral data reported) have been observed in *Rhyacophila nubila* (Zetterstedt) (Rhyacophilidae) (Löfstedt et al., 1994). In both *Rhyacophila* species, sexual communication appears to involve a traditional sex pheromone, with females attracting males. *R. nubila* females produce approximately equal amounts of heptan-2-ol, heptan-2-one, and nonan-2-one, and they produce a smaller amount of nonan-2-ol (although considerable variability was observed, with some extracts predominating in heptan-2-one). *R. fasciata* females produce mainly heptan-2-ol, with smaller amounts of heptan-2-one, nonan-2-one, and nonan-2-ol. Appropriate blends of the syn-

thetic compounds were shown to be attractive to males of *Rhyacophila fasciata*. Ansteeg and Dettner (1991) previously identified heptan-2-ol from females of *R. nubila*, but they did not test this compound for attraction. Males of both these *Rhyacophila* species (Ansteeg and Dettner, 1991; Löfstedt et al., 1994) produce acetophenone, hexanoic acid, and octanoic acid (the possible role of these compounds in sexual communication has not been tested). Females of a third rhyacophilid species, *Rhyacophila fuscula* (Walker), have been shown (Duffield, 1981) to produce nonan-2-ol (similarly, the possible role of this compound in sexual communication has not been tested).

Sexual communication in *Hydropsyche angustipennis* appears to be more complex (Valeur et al., 1990; Löfstedt et al., 1994). Females produce nonan-2-one (the only compound that was electrophysiologically active for male antennae), but males produce a blend of seven compounds (all of which were electrophysiologically active for male antennae) that included heptan-2-one, octan-2-one, nonan-2-one, decan-2-one, undecan-2-one, nonan-2-ol, and (*Z*)-6-nonen-2-one. This blend of male-produced compounds [(*Z*)-6-nonen-2-one was omitted from the blend] attracted both males and females to traps, suggesting that it may act as an aggregation pheromone, not as a traditional sex pheromone. Males and females of *Hydropsyche siltalai* produce the same compounds as the respective sex of *Hydropsyche angustipennis* (Löfstedt et al., 1994).

Mating communication in *Hesperophylax occidentalis* appears to involve a traditional sex pheromone that is produced by females and that attracts males. Female extracts elicited strong electrophysiological activity from male antennae, but not from female antennae, and male extracts did not elicit strong electrophysiological activity from antennae of either sex. The electrophysiologically active compound in female extracts was 6-methylnonan-3-one, and this compound attracted males but not females. This implies that mating communication in *H. occidentalis* is similar to that observed for the *Rhyacophila* species studied by Löfstedt et al. (1994) and does not resemble the system of mating communication observed in *Hydropsyche angustipennis* (Löfstedt et al., 1994).

Although chemical identifications of sex pheromones have not been reported for the Limnephilidae prior to the present study on *H. occidentalis*, a blend of defensive compounds (active against ants) has been chemically identified from the fifth abdominal sternite for both male and female *Pycnopsyche scabripennis* (Limnephilidae), including *p*-cresol, indole, and skatole (Duffield et al., 1977). Similarly, a defensive role was ascribed to compounds found in the fifth abdominal sternites of two other caddisfly species, *Polycentropus flavomaculatus* (Pictet) and *Phryganea grandis* L., on the basis of irritability reactions in larvae of *Calliphora vomitoria* (Ansteeg and Dettner, 1991). *Polycentropus flavomaculatus* (Polycentropodidae) males produce 2-methylbutanoic acid, and both sexes produce several hydroxymethylacetophenones that have not yet been fully characterized (Ansteeg and Dettner, 1991). Female *Phryganea grandis* produce a

blend of pentan-1-ol, octan-1-ol, and 3-methylheptan-2-one, and 2-phenylethanol (Ansteeg and Dettner, 1991).

In our preliminary observations during the present study, we did not observe discrete pheromone glands in the female abdomen. However, we found, by gas chromatographic analysis, that the region between the midpoints of the fourth and fifth sternites in *H. occidentalis* females was the only part of the ventrum that contained the main sex pheromone component 6-methylnonan-3-one. A paired exocrine gland associated with the fourth and fifth abdominal sternites has been identified as the source of pheromonal secretions in *Hydropsyche angustipennis*, *Rhyacophila nubila*, and *R. fasciata* (Löfstedt et al., 1994). There have been reports of pheromone release by Trichoptera from both the fourth and fifth sternites (Resh and Wood, 1985; Solem, 1985; Resh et al., 1987; Wagner and Rosovsky, 1991; Löfstedt et al., 1994). Most female ditrysian Lepidoptera studied have pheromone glands in the region between abdominal segments eight and nine (Götz, 1951; Jefferson et al., 1968; Percy and Weatherston, 1971; Percy-Cunningham and MacDonald, 1987) that may be homologous (Wagner and Rosovsky, 1991) to exocrine glands observed on the eighth and ninth abdominal segments of several Trichoptera (Nielsen, 1980). The postures of pheromone-releasing Lithocolletinae (Tineiodea) and *Niditinea* suggest that the site of pheromone release is also from terminal abdominal segments (Wagner and Rosovsky, 1991). In contrast to higher Lepidoptera, the female genitalia of these species appear to be involved in pheromone release. Although pheromones have not been identified from preditrysian Lepidoptera, sternal glands and globular reservoirs associated with the fifth abdominal sternite have been described for the Micropterigidae (Philpott, 1925; Kristensen, 1984), Heterobathmiidae (Kristensen and Nielsen, 1979), Neopseustidae (Davis, 1975), and Eriocraniidae (Davis, 1978). Wagner and Rosovsky (1991) surmised from these observations that female pheromone-releasing tissues or glands have evolved independently at least five times in the Amphiesmenoptera from structures on the thorax and abdomen.

The active compounds released by many species of Lepidoptera are straight-chain aliphatic molecules (12–18 carbon atoms) with a single functional group (alcohol, aldehyde, epoxide, ketone, etc.) and one or more double bonds (Arn et al., 1986; Tamaki, 1988; Mayer and McLaughlin, 1991). Recently, small (7–9 carbon atoms) secondary alcohols and ketones have been identified from some species of Lepidoptera (Tóth et al., 1995; Zhu et al., 1995). These compounds are similar in structure to the ones that have been implicated in pheromone communication in *H. occidentalis* (present study) and other caddisfly species (Löfstedt et al., 1994). This structural similarity appears to reflect the close phylogenetic relationship of the Trichoptera and Lepidoptera and may represent a symplesiomorphy for these sister groups, as suggested by Zhu et al. (1995).

Resh and Wood (1985) suggested that understanding chemical communication in the Trichoptera may provide insights into their systematic and ecological relationships. The Trichoptera are one of the largest and most successful groups of aquatic insects (Wiggins and Mackay, 1978), with approximately 1340 Nearctic species for which the adult systematics have been well established (Duffield, 1981; Wiggins, 1988). The Trichoptera and the Lepidoptera constitute a monophyletic group, the superorder Amphiesmenoptera, which is among the most highly corroborated ordinal clusterings in the Insecta (Hennig, 1953; Kristensen, 1981a, b, 1984; Nielsen, 1989). Similarities in morphology (Ross, 1955; Carpenter, 1992), embryology (Kobayashi and Ando, 1988), and pupation (Weaver, 1984; Wiggins and Wichard, 1989; Wiggins, 1992) have been found to support a strong phylogenetic relationship between the two orders. The time of reproductive activity is also similar for the Trichoptera and for the Lepidoptera. Adults of many caddisfly species are reproductively active following eclosion at either dusk or dawn (Davis, 1934; Solem, 1986; Peckarsky et al., 1990), and their activity is dependent on light intensity, but not temperature (Benz, 1975). Similarly, flight activity in early Lepidoptera (such as the Hepialidae) is dependent on light intensity (Madge, 1954; Martyn, 1960; Barton Browne, et al. 1969; Van Gerwen et al., 1972; Hardy, 1973; Mikkola, 1974; Mallet, 1984; Wagner, 1985). In trichopteran families such as the Lepidostomatidae and the Sericostomatidae, the basal segments of the male antennae are enlarged and distorted (Wiggins, 1984). This is suggestive of the sexual dimorphism associated with sex pheromone reception in male saturniid moths (Boeckh, 1967; Lerner et al., 1990), in which the larger male antennae are important for long-distance pheromone reception (Steinbrecht, 1987).

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Hesperophylax occidentalis (TRICHOPTERA: LIMNEPHILIDAE): ELECTROANTENNOGRAM STRUCTURE-ACTIVITY STUDY OF SEX PHEROMONE COMPONENT 6-METHYLNONAN-3-ONE

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Abstract—Electroantennogram (EAG) analyses of *Hesperophylax occidentalis* male and female antennae were used to generate dose-response curves for synthetic, racemic 6-methylnonan-3-one and to demonstrate the chemical specificity of male antennal olfactory cells. Male antennae responded to 6-methylnonan-3-one, the main female pheromone component. Females also demonstrated a response to 6-methylnonan-3-one, but a smaller one than males. The chemical specificity of male antennal receptors was determined by comparing EAG activity of synthetic, racemic 6-methylnonan-3-one and a series of structural analogs. The three structural parameters considered, including keto position, chain length, and methyl-group position, were demonstrated to contribute significantly to the reception of synthetic, racemic 6-methylnonan-3-one by male antennae. For straight-chain nonanones with keto groups at different positions, nonan-3-one elicited the greatest EAG responses from male antennae. For straight-chain alkan-3-ones with different chain lengths, the EAG responses of male antennae to nonan-3-one and decan-3-one were the same, but greater than those to other chain lengths. For methyl-branched nonan-3-ones, 6-methylnonan-3-one elicited greater EAG responses from antennae than analogs with the methyl group at another position.

Key Words—Caddisfly, limnephilid, aquatic insect, adults, EAG, attractant, antennae, analogs, chemical specificity, receptors.

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INTRODUCTION

Mayer and McLaughlin (1991) listed over 1300 insect species for which the sex pheromones or other attractants have been reported. These species comprise 10 orders, including the Trichoptera (Jewett, 1993; Löfstedt et al., 1994; Bjostad et al., 1995), Orthoptera, Homoptera, Hemiptera, Lepidoptera, Coleoptera, Mecoptera, Neuroptera, Hymenoptera, and Diptera (Mayer and McLaughlin, 1991). Despite its pervasiveness, the use of sex pheromones by members of the Trichoptera and other aquatic insects has received little attention until recently.

Wood and Resh (1984) first demonstrated chemically mediated sexual communication in the Trichoptera by trapping *Gumaga griseola* males with live, caged females and whole-body extracts. Chemical extracts of specific body sections from adult *G. nigricula*, *G. griseola*, and *Dicosmoecus gilvipes* females have also been used in field trapping (Resh and Wood, 1985). These experiments indicated that the fifth abdominal sternite of females contains the sex pheromone. Similar methods were employed to demonstrate sex pheromone use by the rhyacophilid, *Rhyacophila nubila* (Solem, 1985).

The identification of sex pheromones has been reported for several caddisfly species, including *Hesperophylax occidentalis* (Jewett, 1993; Bjostad et al., 1995), *Hydropsyche angustipennis* and *R. fasciata* (Löfstedt et al., 1994). *H. occidentalis* females produce a blend of compounds to which males demonstrate both an electrophysiological response in the laboratory and a behavioral response in the field (Jewett, 1993; Bjostad et al., 1995). Previous electroantennogram (EAG) experiments demonstrated that *H. occidentalis* males respond to extracts of the membrane between the fourth and fifth sternites in *H. occidentalis* females, but not to the sternites themselves. The pheromone blend was shown to include indole, 6-methylundecan-3-ol, 6-methylnonan-3-ol, 6-methylnonan-3-one, 6-methyloctan-3-ol, 6-methyloctan-3-one, 6-methylheptan-3-ol, and 6-methylheptan-3-one. Of these compounds, 6-methylnonan-3-one is present in greatest abundance and elicits the strongest EAG response from male antennae (Jewett, 1993; Bjostad et al., 1995).

The electroantennogram (EAG) has been a tool frequently used to study antennal receptor function for sex pheromones of moths and other insects. EAG response is assumed to be the summation of bioelectrical potentials generated by many olfactory receptor cells responding to odor stimulants (Nagai, 1984). The amplitude of the receptor potential of most insects is influenced not only by stimulus intensity, but by the chemical structure of the stimulus. The intersexual exchange of information by insects often transpires via molecules that differ only by their functional groups (Bestmann et al., 1987). Although receptor neurons are generally most active when exposed to the genuine pheromone, individual neurons may be excited by higher doses of a related analog. Male antennal neurons of many lepidopterans are generally receptive to pheromones

released by conspecific females or females belonging to closely related sympatric species (Priesner, 1979).

Interactions between pheromone molecules and their receptors have been investigated by analyzing the responses of identified receptor neurons to a homologous series of compounds (Boeckh, 1967; Kafka, 1970, 1974; Liljefors et al., 1985; Priesner et al., 1977; Schwarz et al., 1989; Vareschi, 1971; Dumpert, 1972; Kafka and Neuwirth, 1975). The literature is replete with structure–activity studies for lepidopteran, hymenopteran, orthopteran, isopteran, dipteran, and coleopteran pheromones (Prestwich, 1987). Analogs have been classified by chain length, functional group, regiochemicals with relocated double bonds, or carbonyls, diastereoisomers, and enantiomers (Prestwich, 1987).

The chemical specificity of *H. occidentalis* male antennae for 6-methylnonan-3-one was evaluated by comparing its EAG activity with that of several analogs. The analogs tested were different from 6-methylnonan-3-one either in keto position, chain length, or methyl-group position.

METHODS AND MATERIALS

Collection of Pupae and Preparation of Adults. Pupae were obtained during the summer and early autumn of 1990 and 1991 from a small stream in Hewlett Gulch, a tributary of the Poudre River (Larimer County, Colorado), or from the westernmost lake at Glacier Lakes approximately 5 km northwest of Centennial, Wyoming. Pupae in larval cases attached to the undersides of rocks by a silk thread were removed and placed into insulated coolers (4-liter capacity; Rubbermaid, Gott Corp., Winfield, Kansas) containing ambient water. Sufficient aeration was provided by battery-powered aquarium pumps (B&M Air Pump, West Point, Mississippi 39773) similar to those used by fishermen to maitain live bait. Pupae were returned to the laboratory where preparations were made for their eclosion.

Adult females frequently emerged several days later than males, but overlapping was observed. Behavioral studies suggest that caddisfly adults may be reproductively active following eclosion at either dusk or dawn (Williams and Feltmate, 1992). To prevent copulation and its consequent effects upon female production of or male response to pheromone that has been observed in some insects (McNeil, 1991), adults were removed from their styrofoam emergence containers immediately after eclosion and separated by sex.

The sexes of *H. occidentalis* adults were determined using their genital characteristics, abdominal color, and/or odor. Under a 25× low-power microscope, claspers that males use during copulation are visible. Parker and Wiggins (1986) present excellent illustrations of male and female genitalia. Abdomens of males were usually a pale green, while females could be identified by their

drab brown color. This characteristic is subject to geographical variation (Parker and Wiggins, 1985). Finally, the odor of compounds (to the human nose) secreted from a female's abdomen was very distinct, while the male displayed no detectable odor. Voucher specimens were deposited in the C.P. Gillette Museum of Arthropod Biodiveristy at Colorado State University, Fort Collins, Colorado 80523.

Synthetic Compounds. Octan-3-one, nonan-3-one, decan-3-one, and nonan-2-one were purchased from Aldrich Chemical Company, Inc. (1001 St. Paul Avenue, Milwaukee, Wisconsin 53233) (Figure 1). Nonan-4-one and 2-methylnonan-3-one were purchased from Wiley Organics (1245 6th St., Coshocton, Ohio 43812) (Figure 1). Dr. Louis Hegedus (Chemistry Department, Colorado State University) provided the nonan-5-one (Figure 1).

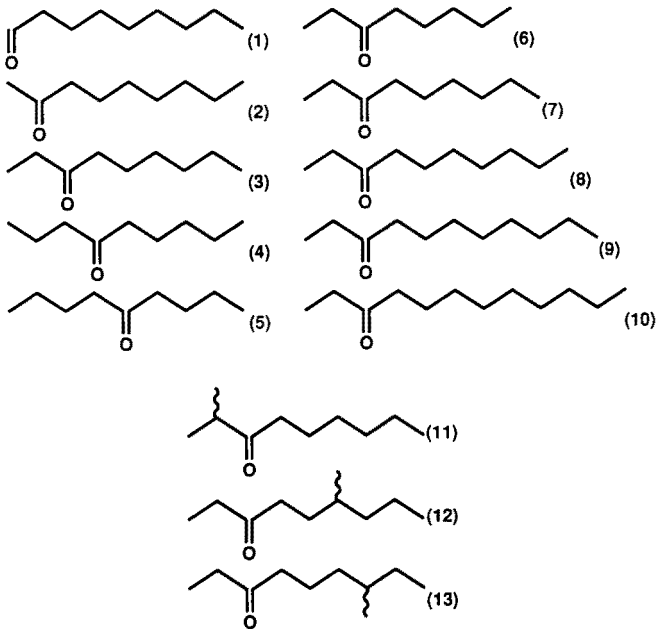

FIG. 1. Synthetic compounds used to characterize the specificity of male *Hesperophylax occidentalis* antennae. Squiggly lines represent a methyl group of undetermined stereochemistry; (1) nonanal, (2) nonan-2-one, (3) nonan-3-one, (4) nonan-4-one, (5) nonan-5-one, (6) octan-3-one, (7) nonan-3-one, (8) decan-3-one, (9) undecan-3-one, (10) dodecan-3-one, (11) 2-methylnonan-3-one, (12) 6-methylnonan-3-one, and (13) 7-methyl nonan-3-one.

The optical nature of the main sex pheromone component, 6-methylnonan-3-one, in *H. occidentalis* females is presently unknown. Many lepidopteran pheromones are optically active (Mayer and McLaughlin, 1991), which suggests a role for stereospecificity in the pheromone blend of *H. occidentalis* females. However, we did not have access to different optical isomers of 6-methylnonan-3-one, and only racemic mixtures were tested.

The racemic compounds 6-methylnonan-3-one, and 7-methylnonan-3-one (Figure 1) were synthesized from the racemic starting compounds 4-methylheptan-1-ol and 5-methylheptan-1-ol (Wiley Organics), respectively. Dodecan-3-one, and undecan-3-one were synthesized from decan-1-ol (Aldrich Chemical Company, Inc.), and nonan-1-ol (Aldrich Chemical Company, Inc.), respectively. Syntheses were completed by oxidizing 4-methylheptan-1-ol, 5-methylheptanol, decanol, and nonanol with pyridinium dichromate (PDC) (Corey and Schmidt, 1979) in $MeCl_2$ to form the corresponding aldehydes. A Grignard reaction with ethyl magnesium bromide (EtMgBr) in diethyl ether (Et_2O) was then performed to generate the secondary alcohols (Fieser and Fiesser, 1967). Reaction with EtMgBr was followed by another oxidation by PDC to generate the ketones. Final purification of the ketones was accomplished by their elution through a Florisil column with an equimolar mixture of $MeCl_2$ and Et_2O.

Synthesis of the racemic compounds 6-methylnonan-3-one, 7-methylnonan-3-one, dodecan-3-one, and undecan-3-one was modified from Corey and Schmidt (1979) and Fieser and Fieser (1967). The PDC reaction proceeded for 24 hr instead of 1 hr, with stirring provided by a 5-mm metal-impregnated glass rod. The remaining solid material was centrifuged to a small pellet at the bottom of the vial, and the final product in solution was transferred with a pipet to a clean vial.

Workup of the Grignard reaction was also modified. Excess EtMgBr was destroyed by adding to the reaction medium a water-saturated Et_2O solution. Destruction of excess EtMgBr was complete when water was no longer observed to react with it. The remaining white solid material was centrifuged to a pellet, and the Et_2O solution was pipetted to a clean vial, where it was extracted several times with distilled water. The final product was passed through a column of Florisil packed with Et_2O.

Treatment Preparations. Dose–response curves for males and females were obtained with five amounts of 6-methylnonan-3-one in $MeCl_2$: 0.1, 1.0, 10.0, 100, and 1000 μg, and a blank of 100 μl $MeCl_2$ that was evaporated to apparent dryness before testing.

In studies using synthetic analogs with different chain lengths, different keto positions, or different methyl-group positions, units of 200 μg were tested. All experiments included comparisons with 200 μg of synthetic, racemic 6-methylnonan-3-one as a positive control, and a blank of 100 μl $MeCl_2$ that

was evaporated to apparent dryness before testing. Preliminary experiments indicated that 200-μg doses elicited the maximum EAG response possible for each compound (for example, see Figure 2 below), which would tend to minimize differences in EAG responses due to differences in volatility among the compounds tested.

EAG Procedure. Because their life has not been observed to exceed five days under laboratory conditions, expedient use of the adult caddisflies was important. One antenna held at the base was plucked from the head with a pair of fine forceps. To increase exposed surface area and facilitate measurement of any depolarization, a 0.5-cm tip of the antenna was removed with a pair of thread trimmers and discarded. The remainder of the antenna was placed between two glass pipets filled with 0.15 M potassium chloride solution serving as electrodes. A few minutes were frequently required before testing to permit equilibration of the antenna and the KCl solution. Treatments were applied to separate 1×3-cm strips of filter paper from which the $MeCl_2$ was permitted to evaporate. The filter paper was folded lengthwise, slipped into a Pasteur pipet, and used immediately before active compounds could volatilize. Treatments were tested in random order. A 5.0-ml glass syringe was used to expel 0.5 ml air through the Pasteur pipet and across the antenna. A drop in potential across an antenna was enhanced by a $5000\times$ amplifier interfaced with a Commodore 64 computer (Commodore Business Machines, Wayne, Pennsylvania), and displayed graphically on a monitor (Bjostad and Roelofs, 1980; Bjostad, 1988). Fatigue was minimal throughout the duration of testing each antenna, as indicated by the small variation in responses to compounds tested.

Statistical Analyses. Data obtained from the electroantennograms were analyzed with SAS (SAS Institute Inc., SAS Circle, Box 8000, Cary, North Carolina 27512-8000). Experiments in which ANOVA yielded significant P values (0.05) were subjected to a comparison of total EAG activity with Newman-Keuls multiple-range test.

RESULTS

Both male and female antennae produced significant EAG responses to synthetic, racemic 6-methylnonan-3-one (Figure 2). The EAG responses of males to synthetic, racemic 6-methylnonan-3-one were greater than the EAG responses of females. Electrophysiological responses of both male and female antennae were directly proportional to the amount of synthetic, racemic 6-methylnonan-3-one applied to them (Figure 2). The amount of 6-methylnonan-3-one required to elicit a response from male antennae was four orders of magnitude less than that required for females; 1000 μg of 6-methylnonan-3-one was required to elicit a response from female antennae significantly greater than that elicited by a

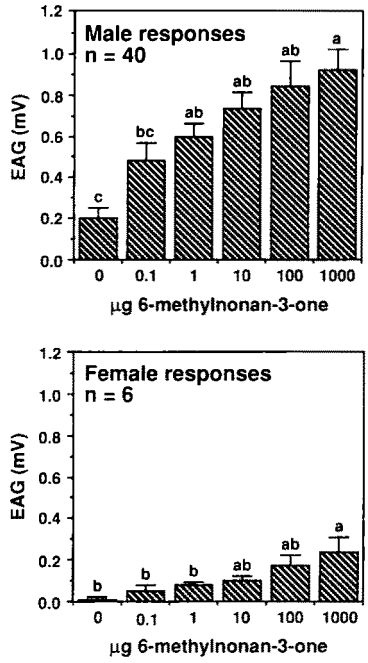

FIG. 2. Synthetic, racemic 6-methylnonan-3-one dose–response curves for *Hesperophylax occidentalis* males and females; n = number of responses to each treatment recorded from three male and three female antennae. Bars on columns represent standard errors. Treatments denoted by the same letter are not significantly different by Newman-Keuls multiple-range test ($P = 0.05$).

control of evaporated $MeCl_2$. The lowest dose of 6-methylnonan-3-one tested was 0.1 μg, and it elicited a significantly greater response from male antennae than the control.

Results of the EAG experiments were consistent with our prediction of chemical specificity for reception of 6-methylnonan-3-one by male antennal olfactory cells and support the importance of all structural parameters used. The responses elicited from male antennae waned with analogs whose structure deviated increasingly from that of the main pheromone component, 6-methylnonan-3-one. When considering the effect of different keto positions, there were also significant differences in the EAG activity elicited by analogs of 6-methylnonan-3-one (Figure 3). Multiple-range tests indicated that a significant difference exists between nonan-3-one and all other treatments, but no statistically significant differences exist among nonan-2-one, nonan-4-one, and nonan-5-one.

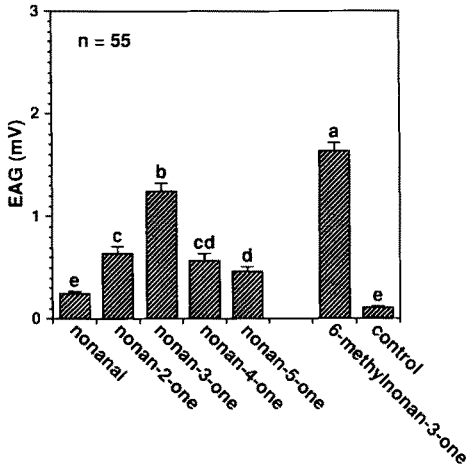

FIG. 3. *Hesperophylax occidentalis* male EAG responses to nonanones with varying keto positions (200 μg of each); n = number of responses to each treatment recorded from 11 male antennae. Bars on columns represent standard errors. Treatments denoted by the same letter are not significantly different by Newman-Keuls multiple-range test (P = 0.05).

Significant differences were obtained between nonan-5-one and nonanal and between nonanal and the control of evaporated $MeCl_2$. Responses elicited by 6-methylnonan-3-one were significantly different from all other treatments.

When considering the effect of different chain lengths, a significant difference among EAG activity elicited by analogs of 6-methylnonan-3-one was observed (Figure 4). EAG responses of male antennae to nonan-3-one and decan-3-one were equally strong. These two compounds elicited significantly higher responses than octan-3-one, which elicited significantly higher responses than undecan-3-one. Undecan-3-one elicited significantly higher responses than dodecan-3-one. No significant difference was observed between EAG responses to dodecan-3-one and the control of evaporated $MeCl_2$. The average response elicited from male antennae by 6-methylnonan-3-one in that experiment was significantly greater than all other treatments.

Significant differences were observed among the EAG activity of 6-methylnonan-3-one analogs differing in methyl-group position (Figure 5). Responses by male antennae to methyl-group position analogs were smaller than those of synthetic 6-methylnonan-3-one. Furthermore, the magnitude of the EAG responses was inversely related to the distance of the methyl group from the sixth carbon. 7-Methylnonan-3-one elicited a significantly larger response than

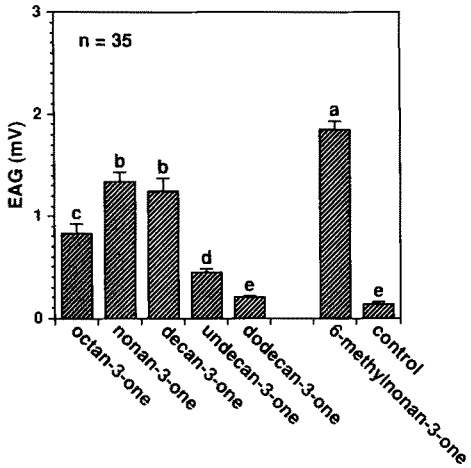

FIG. 4. *Hesperophylax occidentalis* male EAG responses to alkan-3-ones of varying length (200 μg of each); n = number of responses to each treatment recorded from 11 male antennae. Bars on columns represent standard errors. Treatments denoted by the same letter are not significantly different by Newman-Keuls multiple-range test (P = 0.05).

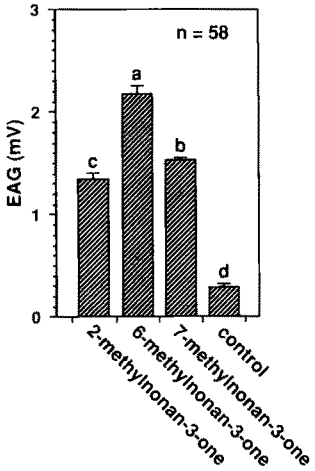

FIG. 5. *Hesperophylax occidentalis* male EAG responses to varying methyl-substituted nonan-3-ones (200 μg of each); n = number of responses to each treatment recorded from 12 male antennae. Bars on columns represent standard errors. Treatments denoted by the same letter are not significantly different by Newman-Keuls multiple-range test (P = 0.05).

2-methylnonan-3-one. Other commercially available methyl-branched alkanones were also tested as indicators of antennal specificity for the main pheromone component. 7-Methylnonan-3-one and 2-methylnonan-3-one elicited significantly larger EAG responses than 5-methyloctan-3-one. 5-Methyloctan-3-one elicited a response significantly higher than that elicited from 4-methylheptan-3-one. The activity elicited by 4-methylheptan-3-one was not significantly different from the control. The average male EAG response elicited by synthetic 6-methylnonan-3-one was significantly greater than that from all other treatments.

DISCUSSION

Results of our EAG experiments were consistent with previous observations that females produce compounds to which males have a highly selective response. Small structural changes in the molecule 6-methylnonan-3-one caused appreciable reductions in the receptor potentials of *H. occidentalis* male antennae. These reductions in receptor potential demonstrate that the olfactory cells of male antennae are selective for 6-methylnonan-3-one. The structural parameters demonstrated to be responsible for this selectivity are keto position, chain length, and methyl-group position. The consequences of selectivity by olfactory cells are illustrated by our experiment comparing the electrophysiological activity of chain length analogs. EAG responses of male antennae to nonan-3-one and to decan-3-one were the same, consistent with our expectation that the putative receptor for 6-methylnonan-3-one is specialized for a compact molecule that is sterically intermediate between nonan-3-one and decan-3-one.

Previous work has demonstrated that similar structural alterations in pheromone components significantly reduce EAG activity, indicating that receptors on insect antennae characteristically have limited flexibility. For sex pheromones of moths, it has been demonstrated that chain length, double-bond position, double-bond configuration, and terminal functional groups (typically alcohol, aldehyde, or acetate) all influence the magnitude of EAG responses (Roelofs, 1984). Structure–activity studies with analogs of (*E*,*Z*)-2,13-octadecadien-1-yl acetate, a main pheromone component of the currant clearwing moth, *Synanthedon tipuliformis*, indicate that hydrophobic, steric, and electronic effects of chain terminal groups are important physical parameters responsible for biological activity of the compound (Hoskovec et al., 1993).

Chain length of sex pheromone analogs was an important determinant of EAG activity in our work with *H. occidentalis* and has been considered elsewhere. Electrophysiological recordings from single receptor cells were obtained from antennae of *Agrotis segetum* in response to chain-length analogs of the main sex pheromone component (*Z*)-5-decan-1-yl acetate, and strong reductions

in elicited potentials were observed with chain lengths other than 10 carbon atoms (Liljefors et al., 1985). Single-sensillum studies indicated that the terminal methyl group and the acetate group of (*Z*)-5-decenyl acetate interact with highly complementary sites in the receptor cavity (Bengtsson et al., 1990). Furthermore, the magnitude of responses decreased with the elimination of methylene units.

With respect to alkyl substituents, Jönsson et al. (1991a, 1993) found that methyl groups placed at different locations in (*Z*)-5-decenyl acetate resulted in a significant reduction of EAG response. Methyl-substituted analogs of the pheromone for the turnip moth were synthesized and used to study the degree of steric complementarity between the acetate-substituted alkyl chain of the pheromone component and its receptor (Jönsson et al., 1991a,b). Fescemeyer and Hanson (1990) introduced either a cyclopropyl or *tert*-butyl group at the terminal portion of (*Z*)-11-tetradecen-1-ol acetate, the main pheromone component of the European corn borer moth *Ostrinia nubilalis*, and they found that EAG responses of males were either significantly higher or lower than responses to the main pheromone component itself, depending on the genetic strain tested.

We have not yet determined the enantiomeric blend of 6-methylnonan-3-one that is involved in pheromone communication in *H. occidentalis*. The EAG technique can play an important role in determining relative antennal response activity among optical isomers that cannot be characterized with minute quantities of natural material (Roelofs, 1984). EAG studies demonstrated that the highest responses occurred selectively with the correct enantiomer of 4-methylheptan-3-ol, the trail pheromone of the ant *Leptogenys diminuta* (Kern and Bestmann, 1993). Studies with the American cockroach *Periplaneta americana* indicated that EAG responses were higher with the correct enantiomer of periplanone-A than with an optically active analog (Okada et al., 1990). *R* and *S* enantiomers of methyl-substituted compounds elicited different EAG responses from male *Agrotis segetum* for some methyl-group positions, but not all (Jönsson et al., 1993). EAGs were used to provide insight into the enantiomeric blend of the aggregation pheromone used by both *Pissodes strobi* and *P. nemorensis* (Hibbard and Webster, 1993). In that study, responses of *P. nemorensis* antennae to (1*R*,2*S*)-grandisal were significantly higher than to (1*S*,2*R*)-grandisal. EAG analyses demonstrated that the 9*S*,10*R* enantiomer of (*Z*,*Z*)-3,6-*cis*-9,10-epoxyheneicosadiene elicits a male antennal response two to three times greater than the 9*R*,10*S* enantiomer from *Hyphantria cunea* and *Estigmene acrea* (Hill et al., 1982).

Kentaro et al., (1991) studied the EAG responses of cockroaches to periplanone and four groups of analogs. The analogs were those containing a germacrene skeleton, those without the *exo*-methylene and isopropyl groups on the 10-membered ring, compounds similar to periplanone, but with altered chain length, and the four candidates of periplanone-A (P-A) including Hauptmann's

P-A, Macdonald's P-A, Persoon's P-A, and its epimer. Hauptmann's P-A elicited the strongest responses of the four analogs, suggesting that it is a natural P-A produced by female cockroaches.

EAGs provide useful information on the specificity of insect olfactory receptors, but recordings from individual olfactory cells are necessary for a detailed understanding of the perception of odor molecules by insects (Wadhams, 1984). For example, in a comparison of EAG responses and single sensillum responses of the sugar beet moth (*Scrobipalpa ocellatella*), Renou et al. (1980) found that the responses to a series of pheromone analogs were almost identical, but the single sensillum recordings showed that responses from two cell types contributed to the EAG profile. Similar specificity of single cells for one compound (or a small set) has been found in other species as well. Single-unit recordings from sex pheromone antennal receptors in the moth *Antheraea polyphemus* indicated that one antennal receptor type responds to the acetate pheromone component, but not to the aldehyde component, and a second antennal receptor responds selectively to the aldehyde component (Bestmann et al., 1987). Single-sensillum recordings from *Yponomeuta rorellus* males revealed two types of cells in most sensilla (Löfstedt et al., 1990). A large spike amplitude cell was stimulated by tetradecyl acetate and the unsaturated analogs (*E*)-11-, (*E*)-6-, and (*E*)-12-tetradecenyl acetate, and to a lesser extent by the (*Z*)-11, (*Z*)-6, and (*Z*)-12 isomers. A cell with medium spike amplitude was stimulated by (*Z*)-9-tetradecenyl, and (*Z*)-11-hexadecenyl acetate. Some sensilla contained a third cell firing with a small spike amplitude, which was activated by (*Z*)-11-tetradecenol.

The dose–response curve in the present study generated from electrophysiological recordings from females with synthetic, racemic 6-methylnonan-3-one supports previously observed female response to female-produced compounds (Jewett, 1993; Bjostad et al., 1995). The response appears to be genuine, and similar scenarios have been observed in lepidopteran species such as *Choristoneura fumiferana* (Birch, 1977; Light and Birch, 1979), and *Trichoplusia ni* (Palaniswamy and Seabrook, 1978). The behavioral significance of *H. occidentalis* females responding to their own sex pheromone has not been determined.

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FEEDING AND OVIPOSITION DETERRENT ACTIVITIES OF FLOWER BUDS OF GLOBEMALLOW, *Sphaeralcea emoryi* TORREY, AGAINST BOLL WEEVIL, *Anthonomus grandis* BOHEMAN (COLEOPTERA: CURCULIONIDAE)

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Abstract—The globemallow, *Sphaeralcea emoryi* Torrey, a plant native to Arizona was evaluated as a source of feeding or oviposition deterrents to the boll weevil, *Anthonomus grandis* Boheman. Feeding and oviposition responses of reproductive weevils to the flower buds and artificial diets spiked with dry powder or extracts of the globemallow buds were determined. Boll weevils were deterred from feeding and ovipositing in the flower buds unless the calyxes were removed. Male and virgin female weevils were discouraged from feeding as much as gravid weevils. Secondary chemicals in the flower buds served primarily as feeding deterrents but also prevented oviposition. The concentration of these chemicals was highest in the calyxes of the buds, and potent deterrent activity could be extracted from the calyxes with methanol. Boll weevils were able to perceive the deterrents by contact chemosensory organs on the antennae, maxillary palps and labial palps.

Key Words—*Anthonomous grandis* Boheman, boll weevil, *Sphaeralcea emoryi* Torrey, globemallow, feeding deterrent, oviposition deterrent, chemical ecology.

INTRODUCTION

The boll weevil, *Anthonomus grandis* Boheman, develops successfully only on plants of the family Malvaceae including *Gossypium hirsutum* L and *Cienfue-*

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gosia drummondii (Gray) in the United States. Globemallows *Sphaeralcea* spp., perennial shrubs native to Arizona, are recognized as alternate feeding hosts for overwintering weevils (Stoner, 1968) and Cross et al. (1975) reported that, like *Hibiscus* and *Pseudabutilon*, *Sphaeralcea* spp. may serve as marginal hosts for boll weevil. Efforts to discover natural products that might be used to manipulate boll weevil behavior have been focused on the defensive mechanisms of several nonhost plants and alternate host plants (Miles et al., 1987, 1990). Maxwell et al. (1965) and Parrot et al. (1966) found that feeding and oviposition by the boll weevil was reduced on the flower buds of *Hibiscus syiacus* L., an alternate host. This finding led to the identification of unsaturated fatty acids and their methyl esters in the calyxes of the hibiscus that acted as feeding deterrents (Bird et al., 1987).

Significantly less feeding and oviposition by the boll weevil in cotton squares treated with an aqueous extract of a globemallow, *S. emoryi* Torrey were reported (Palumbo et al., 1990), suggesting that flower buds of this plant possess some chemical defensive agent(s) limiting weevil attack and that any factors influencing selection of a feeding site or of feeding behavior must also influence oviposition, i.e., oviposition follows only where feeding is successful.

Our studies investigated whether the buds of *S. emoryi* contain feeding and/or oviposition deterrents for the weevils, and if so how they perceive such deterrent stimuli.

METHODS AND MATERIALS

Insects. Boll weevils were obtained from the USDA Boll Weevil Research Laboratory at Mississippi State, Mississippi. They were reared on artificial diet pellets (0.8 cm diam. × 2 cm long made of Coax powder) coated with a 1:1 mixture of beeswax and household paraffin (Vanderzant and Davich, 1961; Gast, 1965), under a 16L:8D photoperiod at 25 ± 1°C. Coax is a proprietary feeding stimulant from Buckeye Oil Seed Prod. Co., Fort Worth Texas 76110 and is used to increase acceptability of insect diets.

Plant and Plant Extracts. Flower buds of a globemallow, *Sphaeralcea emoryi* Torrey were collected in Tucson, Arizona, during May 1990 and 1991. They were dried at 60°C, finely chopped in a Waring blender, and stored at −5°C. Some of the flower buds were dissected into three parts consisting of caylx, petals, and anthers, and dried as above. Fresh flower buds were stored at 4°C. Dry powder of whole flower buds was sequentially extracted with 500 ml each of *n*-hexane, acetone, methanol, and distilled water. The organic solvent extracts were concentrated under reduced pressure to near dryness, and the water extract was filtered through Celite 454 prior to lyophilization. Methanol extracts of the dried powders of the calyxes, petals, and anthers were also prepared.

Physical and Chemical Blocking Sensory Organs. Weevils were anesthetized on an ice bed and their antennal, tarsal, and/or mouthpart sensory organs were surgically or chemically blocked. In surgical operations, antennae, maxillary palps, or labial palps were ablated with a finely tapered forceps. Chemical blocking of sensilla on the tarsi or mouthparts was performed under a binocular microscope by contacting these organs for 20 sec with 6 N HCl containing a drop of Triton X-100 in a microcapillary.

Bioassays. One evaluation of boll weevil response to globemallow was carried out with a two-choice test in a 9-cm Petri dish in which a pair of test materials (treatment and control) were placed in parallel in the center. One set of the test material was composed of intact flower buds and flower buds with the calyxes removed. In another set of choice tests, diet pellets were made by substituting the dry powders of calyx, petals, or anthers for the 75% of Coax powder in the basic diet. These were then compared with the basic diet pellets as control. The organic solvent and water extracts of whole flower buds or methanol extracts of each flower organ of the globemallow were applied topically and spread evenly over the surface of basic diet pellets before bioassay. The control basic diet pellets were treated with the solvent alone prior to testing.

Ten reproductive-age weevils of each sex were placed in a dish and given a choice among five intact buds and five buds from which the calyxes had been removed. Observations were carried out at 30-min intervals during 3.5 hr with five replicates. The number of weevils on the flower buds and the number of feeding on the buds was recorded. Artificial diet pellets (treated and untreated) were also placed in parallel in the center of the dishes and exposed to 10 pairs of reproductive weevils for 20 hr. Male and virgin female weevils were also examined separately in the same bioassay. After 0.5, 1.0, 1.5, 2.0, and 20.0 hr, the number of weevils on each diet pellet was counted, and the total number of weevils at each of the five test periods was recorded, but weevils mounting other weevils feeding or ovipositing were subtracted from the data. At the end of the assay (after 20.0 hr) all weevils were removed from the test arena, and the number of feeding and oviposition punctures was counted and the number of eggs in each diet pellet was counted under a binocular microscope after removing the wax coating. A discrimination index (DI) was calculated for the three criteria of each test material according to the formula: $DI = C/(C + T)$, where C and T were the number of weevils, punctures, or eggs on the control diet (basic diet) pellets and test diet pellets, respectively. Thus, significantly higher DI values than 0.5 meant that weevils were able to discriminate treated diet pellets from untreated (control) ones, indicating high deterrency of treated ones.

Another type of bioassay employed was an olfactometer. The Plexiglas olfactometer (Figure 1) consisted of a round main chamber (9 cm diam. × 7 cm in high) and two side tubular arenas (1.0 cm diam. × 10 cm long) connected

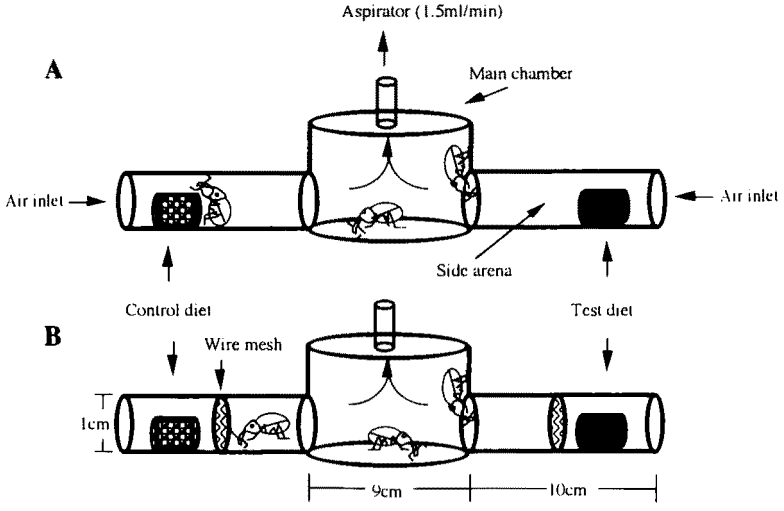

FIG. 1. Diagrams of olfactometers for evaluating behavioral responses of the boll weevil to flower bud extracts of globemallow, *Sphaelarcea emoryi*, in an artificial diet.

with each 40-cm-long glass tube as an air inlet. The two air inlets contained a cotton plug and an activated charcoal filter to purify the ambient air. Ventilation of the chambers (1.5 liters/min) was performed by aspirating air from the center of the lid of the main chamber. Test materials were placed in the side arenas 5 cm from the inner openings. In this bioassay, weevils were allowed access by two different ways to test materials in the side arena. The weevils were, in the first one, able to contact the test material directly (A in Figure 1), and in the second one, a small wire screen was placed just in front of the source of stimuli to prevent the weevils from contacting it (B in Figure 1). The responses of 10 starved weevils per test were observed every 10 min for 1 hr with five replicates, and the number of weevils in each side arena was counted.

All tests were conducted under the same conditions as for adult rearing, and all data were subjected to statistical analysis by χ^2 test.

RESULTS AND DISCUSSION

Feeding and Oviposition Responses to Flower Buds and Plant Extracts. Initial observations were made to detect the ability of boll weevils to feed and/or oviposit on *S. emoryi* flower buds. When the weevils were allowed to choose between the flower buds with and without calyxes in the two-choice test, most

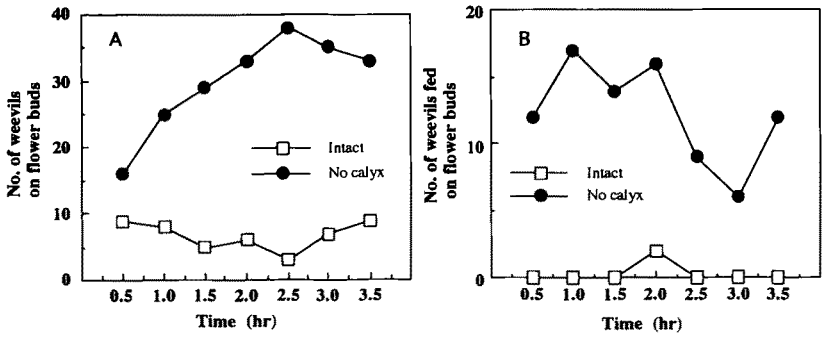

FIG. 2. Comparative responses of boll weevils on globemallow flower buds with and without the calyxes in a two-choice test. (A) Number of weevils on flower buds with calyx (open square) and without calyx (filled circle); (B) number of weevils fed on the flower buds with calyx (open square) and without calyx (filled circle).

were found on the flower buds with calyxes removed, while the number of weevils on intact buds never increased throughout the whole test (Figure 2A). In addition, boll weevils on the two types of flower buds revealed distinctly different behavioral responses. Weevils initiated feeding (puncturing) when they were on the buds with the calyxes removed, but not on the buds with calyxes (Figure 2B). The calyxes of *S. emoryi* were conspicuously pubescent with trichomes. Trichomes on plant surfaces are considered a defensive component of plants against herbivorous insects (Chapman, 1977; Stipanovic, 1983; Southwood, 1986). Trichomes often contain deterrent, repellent, or sticky compounds and can act as an entangling barrier. Palumbo et al. (1990) also observed preferential feeding by boll weevils on pollen through the smooth corolla over the pubescent calyx of *S. emoryi*, and they suggested that the trichomes on the calyx might physically deter females from oviposition.

In the absence of the calyxes (perianth), the developing flower buds of *Hibiscus syriacus*, L. were more acceptable to boll weevils (Parrot et al., 1966; Everett and Earle, 1964) and interference with feeding or oviposition by the calyxes of alternate hosts was effective only at the prebloom stage of the flower buds (Everett and Ray, 1964). We also used normal buds that were completely enveloped in calyxes as intact buds for the bioassays, but some buds partly exposed their petals after the end of the assays, and these were accepted for feeding by the boll weevils.

As shown in Figure 3 a large discrimination index (DI) for the number of weevils, punctures, and eggs was found when the methanol extract of calyxes was applied to the diet pellet surface, and significant deterrent potency to the

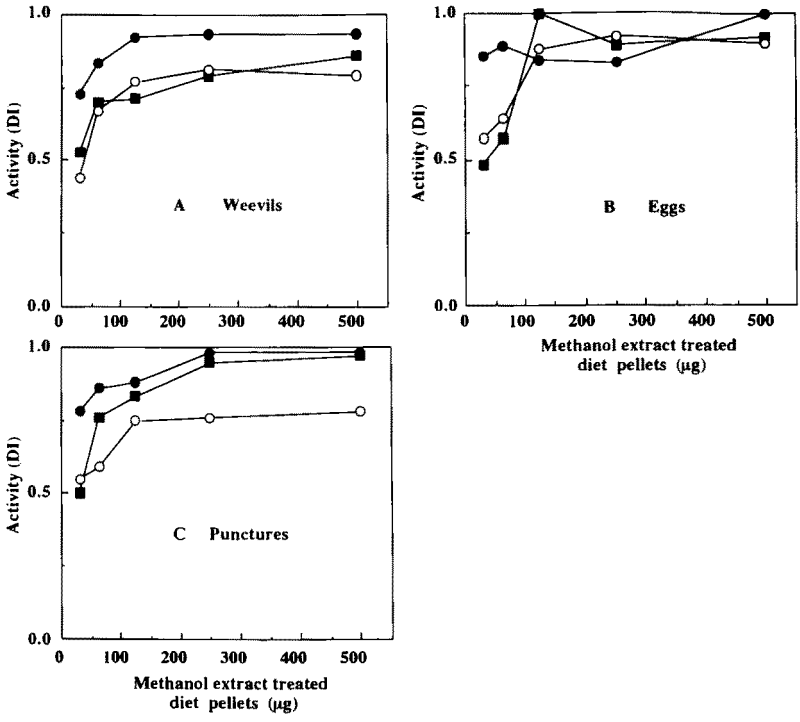

FIG. 3. Globemallow extract deterrent activities evaluated by three criteria: number of weevils (A), eggs (B), and punctures (C) by boll weevil. Methanol extracts of globemallow flower buds were applied on wax coating of artificial diet pellets. Solid square: anther; open circle: petal; solid circle: calyx, $DI = C(C + T)$, C: control; T: test, see text.

weevils was observed even at a dosage of 31.3 μg/pellet. At this dosage, however, methanol extracts of petals and anthers had no deterrent activity, although some deterrency of those extracts occurred at higher dosages. These results suggest the presence of some chemical agent(s) functioning as feeding and/or oviposition deterrents against boll weevils in the calyxes of *S. emoryi* flower buds. The surface of the diet pellets treated with the active calyx extract was slightly sticky compared to those treated with inactive extracts, and it seemed possible that boll weevil feeding or oviposition might have been disturbed by changes in the microtexture of diet pellets. Therefore, a similar preference test was undertaken on artificial diets to which dry powder of flower bud organs was added. When calyx powder or whole bud powder was incorporated into the diet

pellets, the weevils refused to feed or oviposit in these diet pellets, but not in diet pellets containing petal powder or another powder (Figure 4). Under these conditions, the texture of all of the diet pellets was identical. Accordingly, we believe that these studies confirm the presence of chemical deterrents against feeding and/or oviposition by boll weevils in the flower buds of *S. emoryi*. It seems a reasonable defensive strategy for *S. emoryi* to concentrate a deterrent in the calyxes of flower buds, since they constitute the outermost covering of the buds, and constitute an unavoidable barrier when boll weevils attempt to feed or oviposit on the flower buds.

The methanol extract of intact fresh buds at dosages of 500 to 50 μg/pellet evidenced potent deterrent activity to the weevils, while hexane and water extracts were inactive even at 500 μg/pellet (Table 1), demonstrating that methanol was the most effective solvent for extracting the deterrents from the flower buds and that the active component(s) are fairly polar compound(s). It is noteworthy that Palumbo et al. (1990) observed deterrent activity in aqueous extracts of the flower buds of *S. emoryi*, and a feeding deterrent in the calyxes of rose of Sharon, *H. syriacus* L. was water soluble and thermolabile (Maxwell et al., 1965). Several polar unsaturated fatty acids and their esters served as feeding deterrents for weevils in hibiscus (Bird et al. 1987). Clearly, alternative hosts

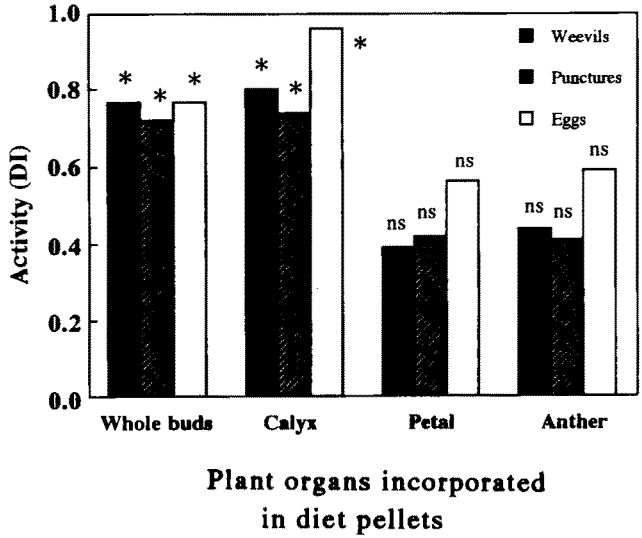

FIG. 4. Discrimination between artificial diets spiked with dry powder of globemallow flower bud organs and unspiked diets. $DI = C(C + T)$, *C:* control; *T:* test; see text, *: Significantly different at $P < 0.05$; ns: not significantly different.

TABLE 1. FEEDING AND OVIPOSITION DETERRENT ACTIVITIES OF *Sphaeralcea emoryi* EXTRACTS TO BOLL WEEVILS

Extracts	Dose (μg/diet)	Activity (DI)[a]		
		Weevils	Punctures	Eggs
Hexane	500	0.45 NS	0.49 NS	0.46 NS
Acetone	500	0.98*	0.96*	1.00*
	100	0.94*	0.84*	0.84*
	50	0.81*	0.62 NS	0.68 NS
	5	0.42 NS	0.50 NS	0.51 NS
Methanol	500	0.98*	1.00*	0.99*
	100	0.76*	0.91*	0.89*
	50	0.40 NS	0.75*	0.80*
	5	0.40 NS	0.50 NS	0.43 NS
Water	500	0.50 NS	0.52 NS	0.45 NS

[a] $DI = C/(C + T)$; C: control, T: Test; see text. * Significantly different at $P < 0.05$ by χ^2 test; NS not significantly different.

of the boll weevil deploy a wide spectrum of defensive feeding deterrents to limit weevil herbivory and reproduction.

Function of Deterrent Agent of Globemallow. When males and virgin females were exposed separately to diet pellets treated with 100 or 500 μg/pellet of the flower bud methanol extract, they completely refused treated ones, yielding large DI values for the presence of weevils and for punctures on the pellets (Table 2). These facts indicated that feeding (puncturing) behavior itself might be affected independently of oviposition because males and virgin females do not lay eggs. In addition, we found no instance where oviposition alone by

TABLE 2. FEEDING RESPONSES OF MALE AND VIRGIN FEMALE BOLL WEEVILS TO METHANOL EXTRACT OF GLOBEMALLOW FLOWER BUDS

Insect	Dose (μg/pellet)	Activity (DI)[a]	
		Weevils	Punctures
Male	500	0.96*	0.97*
	100	0.67*	0.71*
Virgin female	500	0.93*	0.94*
	100	0.83*	0.70*

[a] $DI = C/(C + T)$; C: control, T: test; see text. Data from five replicates.
*Significantly different at $P \leq 0.05$ by χ^2 test

gravid boll weevils was prevented by plant materials or extracts tested in this study. A typical sequence of oviposition behavior by boll weevils consists of six essential behavioral steps including feeding (puncturing), and normal oviposition never occurs on a substrate without feeding (Everett and Ray, 1964). The feeding punctures also serve as the entry point for oviposition. When feeding (puncturing) is negated by a deterrent agent from *S. emoryi*, oviposition was never elicted from females. Therefore, our results clearly demonstrate that a deterrent(s) in the flower buds of *S. emoryi* must act primarily on feeding by the weevils and also serve as a chemical cue preventing oviposition.

Chemosensory Mechanisms of Deterrent Perception. In a preliminary assay, untreated diet pellets (basic diet) and controls (empty) were offered to reproductive pairs of boll weevils in the olfactometer. When allowed to directly access the diet pellets, the starved weevils quickly found the basic diet pellet and accumulated on it. On the other hand, when they were prevented from contacting the diet pellet by a small wire mesh placed in front of the diet pellet, they frequently moved about randomly and went in and out of both side arenas (Figure 5). These results indicate that the weevils must ultimately recognize their feeding and oviposition substrates by contact cues rather than by olfaction. Similar responses by the weevils were observed in the second bioassay, where treated and untreated diet pellets were used as sources of stimuli. When weevils were prevented from contacting the diet pellets, they continued moving in and

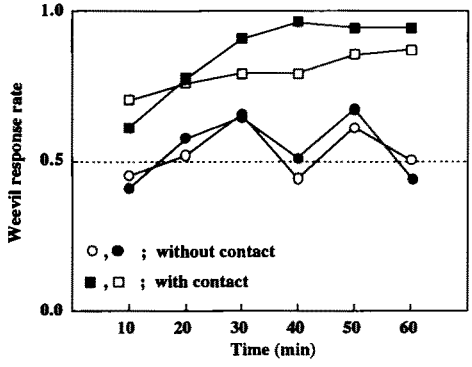

FIG. 5. Discrimination between an artificial diet coated with methanol extract of globemallow flower buds and untreated diet by boll weevils in an olfactometer. Open circle: untreated diet (test) vs. empty arm (control); solid circle: treated diet (test) vs. untreated diet (control); open square: untreated diet (test) vs. empty arm (control); solid square: treated diet (test) vs. untreated diet (control); weevil response is the number of weevils in control side/(number of weevils in control side + number of weevils in test side). Without contact: weevils tested in olfactometer B; with contact: weevils tested in olfactometer A.

out of the side arenas. When they were allowed to contact both diet pellets, they easily discovered and aggregated on the untreated diet pellet. The results of these two olfactometer tests indicate that the flower bud of *S. emoryi* may possess contact deterrent agents but no olfactory repellents.

In the final series of experiments, the sensory basis of deterrent perception was examined by observing modifications in feeding or oviposition behavior induced by selective sensory organ inactivation including those on the antennae, tarsi, and mouthparts. Data are summarized in Table 3. Antennectomy (treatment 1 in Table 3) as well as inactivation of tarsal sensory organs (treatment 2) resulted in DI values near 1, indicating that those treatments were less effective in modifying discrimination between treated and untreated diet pellets. Inactivation of sensilla on the mouthparts with HCl (treatment 3) and palpectomy of the maxillary and labial palps (treatments 4–6) were also unsuccessful in disrupting discrimination behaviors. However, binary blocking (treatments 7–9) by antennectomy and palpectomy resulted in very small DI values, indicating failure of the weevils to discriminate against deterrents. A tertiary blocking (treatment 10) by antennectomy, palpectomy, and tarsal inactivation with HCl was also effective in blocking discrimination, but binary blocking (treatment 11) by

TABLE 3. INFLUENCE OF CHEMICAL AND PHYSICAL BLOCKING OF SENSORY ORGANS ON FEEDING AND OVIPOSITION RESPONSES OF BOLL WEEVILS TO METHANOL EXTRACT OF GLOBEMALLOW FLOWER BUDS

Treatment	Wax coating	Activity (DI)[a]		
		Weevils	Punctures	Eggs
1. Antennectomy	Single	0.88*	0.85*	1.00*
2. HCl blocking of all tarsi	Single	0.86*	0.82*	0.86*
3. HCl block of proboscis	Single	0.92*	1.00*	1.00*
4. Palpectomy in maxilla	Single	0.97*	0.98*	1.00*
5. Palpectomy in labium	Single	0.83*	1.00*	no eggs
6. Palpectomy (4 + 5)	Single	0.91*	0.98*	no eggs
7. 1 + 4	Single	0.61 NS	0.60 NS	0.33 NS
8. 1 + 5	Single	0.58 NS	0.65 NS	no eggs
9. 1 + 6 (4 + 5)	Single	0.42 NS	0.62 NS	no eggs
10. 1 + 2 + 6	Single	0.55 NS	0.60 NS	0.45 NS
11. 2 + 6	Single	0.85*	0.88*	0.90*
12. Intact	Double	0.55 NS	0.45 NS (0.61*)[b]	0.64 NS
13. Control	Single	0.91*	0.80*	0.89*

[a] $DI = C/(C + T)$; C: control; T: test, see text. Data were from three replicates. *Significantly different at $P \leq 0.05$ by χ^2 test.
[b] Number of punctures on inner coating.

inactivating only receptors on the palps and tarsi did not block discrimination. These results suggest that when sensory input from certain organs is blocked, other sensory organs may be able to compensate and allow detection of the deterrents(s).

The weevils were still able to discriminate between the treated and untreated diet pellets when a double coating of paraffin–beeswax was applied to diet pellets in which the inner layer was treated with methanol extract of *S. emoryi* flower buds and the outer layer was untreated (treatment 12). The number of punctures on the outer wax layer was not different from the number on the untreated diet pellets (control), but the number of punctures on the inner wax layer treated with flower bud extract was significantly less than the number of control diets. Under these test conditions, the weevils could make contact with the deterrent agents on the surface of the inner way layer only with mouthparts or ovipositor chemoreceptors, but not with antennal and tarsal chemoreceptors.

In the Coleoptera a variety of sensory organs seem to be involved in host discrimination, although certain ones appear to assume a more dominant or pivotal role. Maxillary palps were shown to be the most important sensory organs affecting egg laying by bean weevils, *Acanthoscelides obtecutus* (Say) (Pouzat, 1970; Szentesi 1976), whereas in Mexican bean beetles, *Epilachna varivestis* Mulsant, antennal and maxillary palp chemosensilla were most responsible for discriminating between susceptible and resistant hosts (Fisher and Kogan, 1976). Our observations of the behavioral responses of boll weevils revealed that they ordinarily contact the surfaces of the test diet pellets with their antennae or mouthparts prior to tarsal contact. Therefore, contact sensory organs on the antennae or mouthparts seems to be important in detecting deterrent agents in the flower buds of the globemallow, *S. emoryi*.

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PHYTOTOXICITY OF SESQUITERPENE LACTONE PARTHENIN ON AQUATIC WEEDS[1]

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Abstract—The sesquiterpene lactone parthenin, one of the major toxins in an obnoxious weed, parthenium (*Parthenium hysterophorus* L.), was toxic at 50 ppm to the floating aquatic weeds pistia (*Pistia stratiotes* L.) and lemna (*Lemna pausicostata* Hegelm.) and at 100 ppm to water hyacinth (*Eichhornia crassipes* Mart Solmns.), salvinia (*Salvinia molesta* Mitchell), azolla (*Azolla nilotica* Decne.), and spirodella (*Spirodella polyrhiza* L. Schleid). The lethal dose for the submerged weeds najas (*Najas graminea* Del.), ceratophyllun (*Ceratophyllum demersun* L.), and hydrilla (*Hydrilla verticillata* L. f. Royle) was 25 ppm. The submerged aquatic weeds were more sensitive to parthenin. Water hyacinth was used as a representative for studying the phytotoxicity of parthenin on aquatic weeds. Inhibition of water hyacinth by parthenin was associated with decline in water use, root dysfunction, excessive leakage of solutes from roots indicative of massive damage to cellular membranes, loss of dehydrogenase activity in the roots, and loss of chlorophyll in the leaves. Plant death occurred in a period of one to two weeks. Parthenin phytotoxicity is gradually lost in an aquatic environment as a lethal dose became nonlethal in about 30 days under outdoor conditions. Possible buildup of a toxin concentration may affect population dynamics and a shift in the aquatic weed flora in the immediate area of parthenium stands. Accumulation of the toxin in an aquatic environment, however, at a level sufficient to produce such changes in a natural ecosystem as a consequence of rain washing parthenium plants and leaching of toxin from their residue appears to be unlikely.

Key Words—Aquatic weeds, parthenin, biomass, membrane integrity, dehydrogenase activity, chlorophyll, water use.

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INTRODUCTION

Parthenium (*Parthenium hysterophorus* L.) is an obnoxious terrestrial weed of worldwide distribution posing serious threats to agriculture, human health, and environment. The weed is well known for its toxic substances. Among the toxic substances are the sesquiterpene lactones parthenin and traces of coronopilin and compounds belonging to phenolic and organic acid classes (Towers et al., 1977; Kanchan and Jayachandra, 1980). The biological activities of sesquiterpene lactones were reviewed by Picman (1986), and the role of terpinoids in allelopathy was discussed by Fisher et al. (1994). Parthenium plants inhibit other plants in their surroundings through inhibitors released from the aerial and underground parts directly and/or from their residue in the soil. Parthenium leaves and inflorescence have been reported to have the maximum quantity of parthenin, followed by the stem and roots (Kanchan and Jayachandra, 1980). This distribution of parthenin coincides with the inhibitory activity of residues of these plant parts (Pandey et al., 1993a,b; Pandey, 1994a,b).

Parthenium residues are relatively more toxic to nine aquatic weeks than to wheat, the test crop species (Pandey et al., 1993a), and to paddy seedling growth (Pandey, 1994b). The present investigation was undertaken to study phytotoxicity of parthenin on nine aquatic weeds to evaluate its possible herbicidal activity on aquatic weeds and to ascertain the implications of parthenin toxicity on population dynamics and shifts in weed flora in a natural aquatic ecosystem.

METHODS AND MATERIALS

Aquatic Weeds. The weeds used in the experiments included the floating weeds water hyacinth (*Eichhornia crassipes* Mart Solmns.), salvinia (*Salvinia molesta* Mitchell), pistia (*Pistia stratiotes* L.), azolla (*Azolla nilotica* Decne.), spirodella (*Spirodella polyrhiza* L. Schleid), and lemna (*Lemna pausiocostata* Hegelm.); and the submerged weeds hydrilla (*Hydrilla verticillata* L. f. Royle), ceratophyllum (*Ceratophyllum demersum* L.), and najas (*Najas gramineae* Del.). All were maintained in the culture medium described earlier (Pandey 1994a).

Isolation and Purification of Parthenin. Parthenin was isolated and purified by the method described by Picman et al. (1980) with modifications (P.V. Subbarao, personal communication, 1992) detailed earlier (Pandey, 1994a). Parthenium leaves were harvested, washed quickly, and blot dried. They were further dried at 70°C for 48 hr. The dried leaves were refluxed with methanol in a Soxhlet apparatus for 12 hr, and parthenin was isolated and purified as previously described (Pandey, 1994a).

Effect of Parthenin on Aquatic Weeds. Parthenin was dissolved in 0.20–0.40 ml of ethanol, distilled water was added to 500 ml, and the final volume made to 1 liter with a nutrient medium (Jain et al., 1989) to prepare solutions of 25, 50, and 100 ppm parthenin. The small quantity of ethanol (maximum final concentration = 0.04%) used for dissolving the toxin did not affect growth of the aquatic weeds, as determined from preliminary results using ethanol at 0.01–4.00%, v/v, in the nutrient medium for culture of the nine aquatic weeds. Final ethanol concentrations that did not inhibit growth of the weeds were 0.10% for azolla and lemna, 2% for najas and hydrilla, 3% for water hyacinth, and 4% (the highest concentration tried) for ceratophyllum, pistia, spirodella, and salvinia. None of the weeds was killed even at 4% ethanol. Plants growing in half-strength nutrient medium served as controls. Ceratophyllum, hydrilla, and najas were grown with and under the stand of floating spirodella and lemna (as direct sunlight was inhibitory to these submerged weeds) in 1-liter beakers with their side walls covered with black paper. Biomass was monitored, and the volume of solutions in the beakers was kept constant by replenishing evapotranspiratory loss twice daily with distilled water.

Physiological Effects of Parthenin Toxicity on Water Hyacinth. Selected physiological effects were studied on water hyacinth plants grown in parthenin solutions. The effect of parthenin on water use by water hyacinth over a brief period was measured by placing preweighed plants in 1 liter of aqueous medium containing 50 or 100 ppm parthenin in half strength nutrient medium (Jain et al., 1989). Plants similarly kept in the medium without parthenin served as controls. Containers of water were also kept for measuring evaporative loss from the free open surface. The plants were removed, weighed, and the volume of aqueous medium was measured at each observation. Then the volume of the medium or water was replenished to 1 liter, and the plants were replaced. The quantity of water absorbed by the plants was calculated by measuring the evaporative loss.

Effect of parthenin on solute leakage from roots of the water hyacinth plants was measured by the procedure of Pandey (1994a). An optical density (OD) of 0.01 at 264 nm (corresponding to UV-absorbing materials such as amino acids, nucleotides, and polypeptides) was considered as one unit of UV-absorbing substances and was expressed on a per gram root (fresh weight) basis.

For total dehydrogenase activity determination, about 0.5 g roots were soaked in 5 ml of 1% (w/v), 2,3,5-triphenyl tetrazolium chloride in darkness at 30°C ± 1°C for 4 hr. The formazan produced was extracted and its absorbance determined at 520 nm (Pandey, 1994a). An enzyme unit was defined as an optical density change of 0.01 OD at 520 nm, and activity was expressed on a per gram root (fresh weight) basis.

Chlorophyll *a* and *b* and total chlorophyll were determined on freshly sampled leaf discs (about 5 cm^2). The discs were weighed and ground in a mortar

with a small quantity of acid-washed silica sand in 25 ml of 80% (v/v) acetone (Pandey et al., 1993a). Absorbance of the final extract was measured at 645, 652, and 663 nm, and chlorophyll *a* and *b* and total chlorophyll, respectively, were calculated considering equal area equal weight by the method of Arnon (1949).

Persistence of Parthenin Phytotoxicity. Pistia was used to assess the persistence of parthenin phytotoxicity, as it was one of the most sensitive species among the floating weeds. Parthenin (50 mg) was dissolved in a small quantity of ethanol and made up to one half the total volume with water and the remaining half with the nutrient medium for a final volume of 1 liter. Preweighed pistia plants were placed in the 50 ppm parthenin solution and incubated for 0, 10, 20, 30, and 40 days under outdoor conditions. Biomass was monitored, and the experiments were conducted under outdoor conditions. All experiments and determinations were repeated at least three times. A completely randomized design was used. The data were subjected to an analysis of variance and when significant *F* values were found, the least significant difference (LSD) was used to separate means.

RESULTS AND DISCUSSION

Results of the effect of parthenin on different aquatic weeds are shown in Figure 1. In controls, all aquatic weeds increased their biomass. Lethal doses of parthenin for different aquatic weeds were 50 ppm for pistia and lemna; 100 ppm for water hyacinth, salvinia, azolla, and spirodella; and 25 ppm each for submerged aquatic weeds ceratophyllum, hydrilla, and najas. The concentrations just below the lethal level reduced biomass in all cases, except in salvinia, in which biomass continued to increase. Higher concentrations of parthenin usually

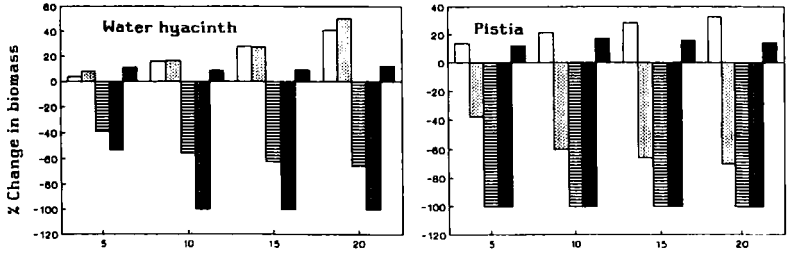

FIG. 1. Effect of parthenin (PAR) on biomass of aquatic weeds. Values are percentage change in biomass over original values and are means of three replications.

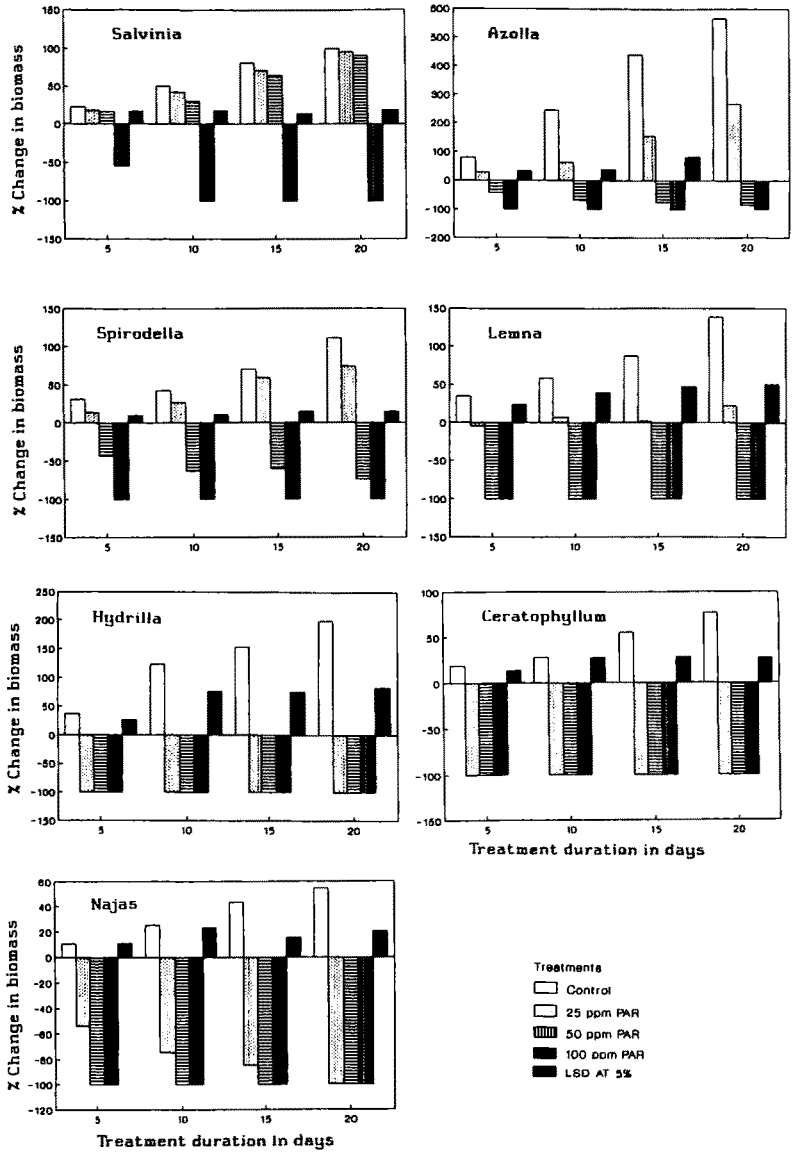

FIG. 1. Continued.

killed plants quicker. Submerged aquatic weeds appear to be more sensitive to parthenin. Parthenin was not inhibitory to water hyacinth at 25 ppm, nor to salvinia even at 50 ppm, although initially short-lived desiccation of frond margins occurred. Parthenin was inhibitory at 25 ppm to pistia > lemna > azolla > spirodella. At 10 ppm the toxin was still inhibitory to the submerged aquatic weeds (data not shown). At the lethal dose, the time required to kill the weeds was about five days for pistia, azolla, lemna, spirodella, hydrilla, and ceratophyllum; 10 days for water hyacinth and salvinia; and 20 days for najas.

The symptoms of parthenin toxicity were drying from leaf or frond margins that gradually progressed towards the growing point, dull green color, flaccid texture, wilting, yellowing, desiccation, and drying of above-water plant parts. The roots showed gradual loss of turgidity, massive fragmentation, and were shed into the medium. These changes consequently resulted in death and decay of the treated plants. Submerged weeds developed flaccid texture, dull green color, and finally fragmentation of plant and plant parts following death and decay.

Parthenin reduced water use by the water hyacinth plants (Figure 2). This was apparent as early as two days after initiation of the treatment. The reduction in water use in treated plants was drastic by six to seven days. The massive increase in solute leakage from the roots of water hyacinth plants grown at the lethal dose of parthenin for two, four, and eight days (Figure 3a) indicated damage to cellular membranes that resulted in loss of cellular structures and

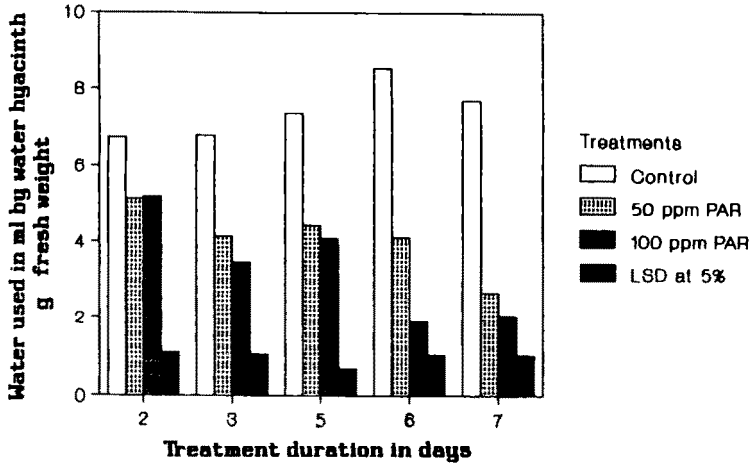

FIG. 2. Effect of parthenin (PAR) on water use by water hyacinth. Values are means of three replications.

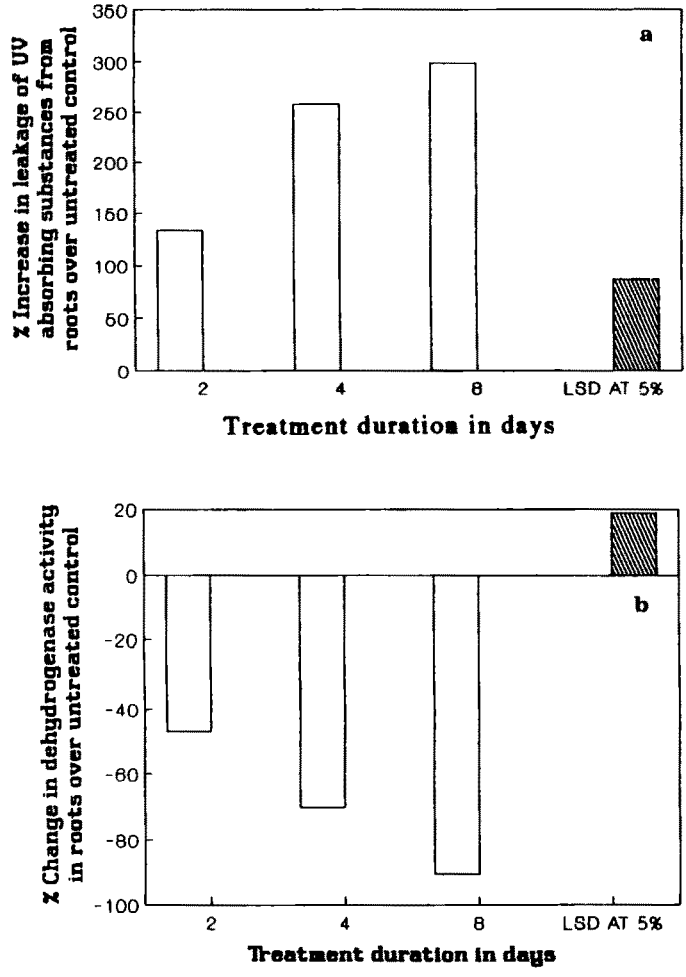

FIG. 3. Effect of lethal dose of parthenin (100 ppm) on (a) leakage of UV-absorbing substance from the roots of water hyacinth and (b) on total dehydrogenase activity in roots of water hyacinth. Values are means of three replications.

constituents. Total dehydrogenase activity in the roots of water hyacinth allowed to grow in 100 ppm parthenin (lethal dose) solution declined rapidly (Figure 3b). Enzyme activity declined by about 50% in two days. Parthenin at the lethal dose markedly reduced chlorophyll *a* and *b* and total chlorophyll contents in water hyacinth leaves (Figure 4).

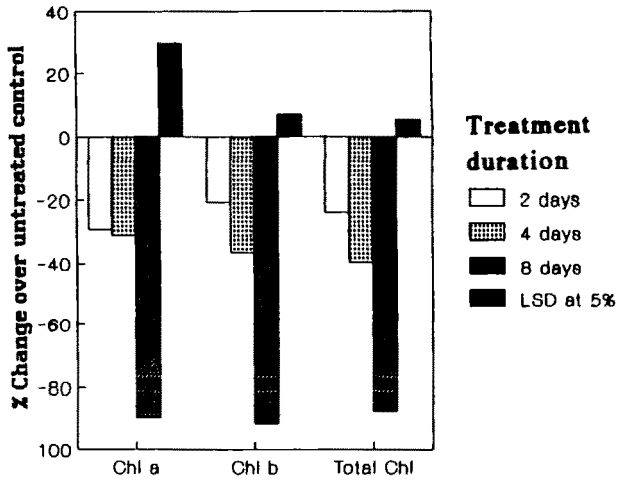

FIG. 4. Effect of lethal dose of parthenin (100 ppm) on chlorophyll *a* (Chl a), chlorophyll *b* (Chl b), and total chlorophyll (Total chl) contents in leaves of water hyacinth. Values are means of three replications.

Parthenin phytotoxicity dissipated rapidly, and a lethal dose for pistia became nonlethal under outdoor conditions in about 30 days, although the medium continued to be inhibitory. After 40 days of incubation of a lethal dose under outdoor conditions, the medium was inhibitory initially for pistia plants, but they recovered and subsequently grew well with an increase in biomass over the beginning value (Figure 5).

Parthenin appears to be a potent phytotoxin in an aquatic environment and might play a decisive role in determining population dynamics and shifts in weed flora in natural ecosystems if it reached concentrations of 25–100 ppm. Submerged weeds appear to be relatively more sensitive to the toxin, and among the floating weeds, pistia was the most sensitive followed by lemna, with all others showing similar sensitivity.

Sesquiterpene lactones have been reported to be phytotoxic. They react with -SH groups of cysteine, glutathione, and many proteins, modifying the original properties of the amino acids and proteins (Picman, 1986). Some are novel uncouplers of oxidative phosphorylation (Chefurka, 1978). The mode of action of parthenin on the weeds in this study is unknown, but a variety of toxic actions, including some of those previously reported, may have combined to cause the stalled growth and development of the weeds in this study. Parthenin reduced water use by water hyacinths and caused desiccation of the above-water plant parts consequent to root dysfunction and death. Massive damage to cellular

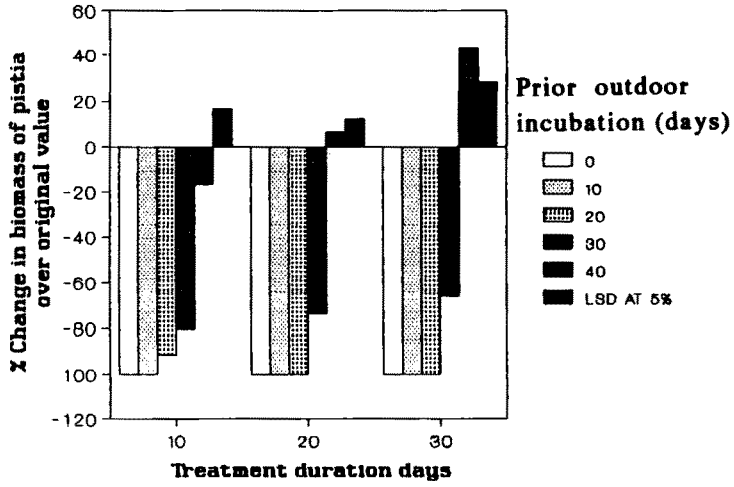

FIG. 5. Persistence of phytotoxicity of parthenin (50 ppm, original lethal dose) under outdoor conditions. Pistia plants were placed in solutions of parthenin (originally 50 ppm) that had been incubated outdoors for 0, 10, 20, 30, and 40 days, and then incubation continued for number of days indicated in the abscissa. Values are means of three replications.

membrane integrity was shown by excessive leakage of solutes from the roots of the treated plants, loss of dehydrogenase activity in roots, and loss of chlorophyll content in the leaves. These effects suggest that parthenin phytotoxicity may be mediated by affecting macromolecules such as proteins, lipids, and nucleic acids. The physiological effects of parthenin in the present study resemble those found earlier with parthenium plant residue on water hyacinth and salvinia (Pandey et al., 1993a; Pandey, 1994a).

Parthenium phytotoxicity dissipated relatively rapidly in about a month under outdoor conditions and was similar to that observed previously (Pandey et al., 1993b; Pandey 1994b). The high level of parthenin in the leaves and flowers largely determines the inhibitory activity of the residue. In a natural ecosystem, build up of concentration of parthenin to a toxic threshold concentration in the area immediately surrounding a parthenium stand may affect population dynamics and shifts in aquatic weed flora, with submerged weeds the first to be affected. Due to the rapid dissipation of parthenin, however, the accumulation and persistence of parthenin in an aquatic environment at a concentration sufficient to produce such changes in natural ecosystems some distance from a parthenium stand appears to be unlikely.

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OVIPOSITION RESPONSE OF *Lobesia botrana* FEMALES TO LONG-CHAIN FREE FATTY ACIDS AND ESTERS FROM ITS EGGS

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Abstract—Avoidance of occupied ovisposition sites supposes that females perceive information related to their own progeny. Fatty acids identified from egg extracts have been reevaluated using a different extraction method, and we have investigated the dose-dependent oviposition response of European grape vine moths (*Lobesia botrana*) to myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, methyl palmitate, methyl oleate, and ethyl palmitate; all except ethyl palmitate have been identified from eggs of *L. botrana*. A methylene dichloride extract of eggs from *L. botrana* revealed the presence of saturated free fatty acids (myristic, palmitic, and stearic) and unsaturated acids (palmitoleic, oleic, linoleic, and linolenic) in amounts ranging from 3.9 ng/egg equivalent for myristic acid to 30 ng/egg equivalent for palmitic and oleic acids. The extract also contained traces of methyl palmitate and methyl stearate. The greatest avoidance indexes were observed in response to palmitic, palmitoleic, and oleic acids. All the other compounds tested caused weaker responses. A reduction in the number of eggs laid was observed when moths were exposed to each of the esters applied at 0.3 μg per application spot. Reduction in eggs laid was also observed at a 10-fold higher dose of oleic acid. The present results confirm that general and simple molecules can be involved in the regulation of oviposition site selection and that they may participate in chemical marking of the eggs.

Key Words—Behavior, *Lobesia botrana*, oviposition, insect, Tortricid, Lepidoptera, fatty acids.

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INTRODUCTION

When females oviposit on nonoccupied plants, but fail to oviposit on similar plants that are already occupied, it may mean that females perceive the occurrence of either eggs, larvae, possible marking of the oviposition site, or plant chemicals induced by larval feeding. Intraspecific avoidance of eggs or larvae exists in several insect species (Anderson, 1986; Roitberg and Prokopy, 1987), and it is assumed that recognition of conspecific individuals would limit competition within species and ensure an optimal share of a resource. It has been suggested that accumulation of individuals in an exhaustible resource may affect the fitness of species (Peters and Barbosa, 1977; Prokopy 1981). Some species probably accrue advantage by avoiding conspecific competitors, but more generally, one may assume that avoiding any potential competitor would be advantageous. A possible way to prevent larval competition, regardless of species, would be for females to respond to general chemical markers. These might be nonspecific chemicals produced by eggs or larvae.

Gas chromatographic analyses of egg extracts revealed the occurrence of common fatty acids (FA) with 14–18 carbons and esters of these fatty acids in several Lepidoptera species (Thiéry and Le Quéré, 1991; Thiéry et al., 1992). Blends of those chemicals deterred oviposition in the tortricid moth *Lobesia botrana*, the European grapevine moth (EGVM) (Thiéry et al., 1992). An identical behavioral response has been reported in three other tortricids: the codling moth (*Cydia pomonella*), the Oriental fruit moth (*Cydia molesta*), and the grape berry moth (*Eupoecilia ambiguella*) Thiéry and Gabel, 1993; Gabel and Thiéry, 1994; Thiéry et al., 1995). In codling moth eggs of different ages, almost constant ratios between FAs, but with important variation in absolute amounts, suggest the hypothesis that FAs may provide some temporal information related to fruit colonization (Thiéry et al., 1995). Oviposition deterrence against *C. pomonella*, *L. botrana*, and *E. ambiguella* was also observed on fruits baited with synthetic blends of fatty acids and esters both in the laboratory and field experiments (Gabel and Thiéry, 1994; Thiéry et al., 1995; and unpublished data). In anticipation of field application, one objective of this study was to verify our previous identification of chemicals extracted from the eggs by washing them in methylene dichloride. Another objective was to focus on the biological activity of the more stable linear fatty acids produced by the eggs, i.e., the saturated ones or those with a single double bond. We also examined the dose-dependent behavioral responses of EGVM females to each of six fatty acids and three esters previously identified from the eggs. The functional perception of fatty acids and esters is discussed in relation to the chemical ecology of several species.

METHODS AND MATERIALS

Insects and Bioassay. Experiments were conducted on 2-day-old *L. botrana* mated females exposed to artificial oviposition substrates (smooth cardboard). Insects (strain from Station Federale de Recherches Agronomiques, Nyon, Switzerland) were reared on semiartificial diet, and adults were maintained after emergence at 23 ± 0.5°C and 85 ± 5% relative humidity during the 16 hr of photophase and at 20 ± 0.5°C and 75 ± 5% relative humidity during the 8 hr of scotophase.

The oviposition assays were conducted with 3 to 4-day-old females grouped by two or three (without males) during one night in Plexiglas tubes (6.5 cm ID, 13.6 cm length) containing a piece of cardboard (10.7 × 13.6 cm) positioned inside the tube at the upper part. Females were isolated the night following exposure in small glass tubes in order to check whether they had started to oviposit. Those that did not oviposit were discarded. Each cardboard provided a dual choice between a 36-cm^2 treated area consisting of 32 equidistant 5-μl application spots each 1.1 cm in diameter, with centers 2.1 cm apart, and a 3× larger non untreated area of 109 cm^2 (Gabel and Thiéry, 1992). An avoidance index (*AI*) was calculated from the numbers of eggs laid on nontreated (*A*) and treated areas (*B*), corrected for the equivalent areas available for oviposition, according to the formula $AI(\%) = [(A - 3B)/(A + 3B)] \times 100$. No avoidance would give a value of 0%, while the *AI* value for maximum avoidance would be 100%. Myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$, *Z*9), oleic acid ($C_{18:1}$, *Z*9), stearic acid ($C_{18:0}$), linoleic acid ($C_{18:2}$, *Z*9–*Z*12), methyl palmitate ($meC_{16:0}$), ethyl palmitate ($etC_{16:0}$), and methyl oleate ($meC_{18:1}$) were obtained from Sigma and were >99% pure. All were tested at doses of 0.3, 1.5, and 3 μg applied in a spot. Palmitic, palmitoleic, and oleic acids also were tested at 0.06 μg/spot. Compounds were diluted according to the dose required in a mixture of acetone–distilled water (5:5) except for stearic acid (acetone–water, 8:2). Doses are given as micrograms per application spot. Their effect was compared to a control (Cs) of acetone–water (5:5) and to paraffin oil (Cw), 3 μg/spot, in acetone–water (5:5). Acetone (purity for GC-MS) and paraffin oil (purity >99%) were from Prolabo.

Chemical Analysis of Egg Extract. Females 2–3 days old were allowed to oviposit on clean glass slides during two nights, until a harvest of ca. 10,500 eggs was obtained. Eggs attached to the glass slides were then extracted for 1 hr in purified methylene dichloride and, after removal of scales by filtration through milipore GW, 0.22 μm, the extract was concentrated under a stream of pure nitrogen. Analysis of the extract was performed by gas chromatography (GC) with no prior treatment except filtration described above and by GC-MS. The GC contained an HP Ultra 1 column (0.20 mm ID × 25 m) and a flame

ionization detector. The temperature program started at 60°C with a 2 min isothermal, and then was linearly increased by 5°C/min to 240°C with a 10-min isothermal at the end. The mass spectrometer was a HP 5971 A mass selective detector with positive 70-eV ionization. Spectra were compared to the MS data bank and to spectra obtained from pure standards. The amount of fatty acids and esters are expressed in nanograms per egg equivalent.

Statistics. In each treatment 12–21 replicates were performed, involving 26–60 females according to treatments. Avoidance indices and numbers of eggs laid were compared between treatments by using the nonparametric Mann-Whitney U test (Siegel, 1956). The null hypothesis that two groups have the same distribution was rejected if $P < 0.01$.

RESULTS

Chemical Analysis of Egg Extract. The GC-MS analysis of the egg extract revealed seven fatty acids, which represented the major constituents. The free acids myristic, palmitic, stearic, palmitoleic, oleic, linoleic, and linolenic acids were present in amounts ranging from 3.9 ng/egg equivalent for myristic acid to 30 ng/egg equivalent for palmitic and oleic acids. Traces of methyl palmitate and methyl stearate were detected but not quantified (Table 1).

Oviposition Preferences. The control solvent (Cs) had no effect on oviposition site selection by EGVM females. The mean avoidance index (AI) in grouped females was 2 ± 2% and was similar in range to that obtained in previous experiments with females grouped by 10 (Gabel and Thiéry, 1992), by five, and by isolated females (Thiéry and Gabel, 1993). Paraffin oil (Cw), which was applied in order to test the perception by females of waxy surfaces, had no effect on the oviposition site selection ($AI = 0 \pm 3\%$, $Z = -2.3$, NS). With Cs and Cw the highest value of *AI* was 7%.

The avoidance response to each of the six fatty acids tested increased with the dose (Figure 1). Palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), and oleic ($C_{18:1}$) acids at 0.06 μg/spot were the most effective acids, and they provoked avoidance in the range of 20%. The avoidance response to $C_{16:0}$ and $C_{16:1}$ acids increased at 0.3 μg/spot ($C_{16:0}$; $Z = -3.31$, $P = 0.001$; $C_{16:1}$; $Z = -4.55$, $P = 0.0001$), and the maximum response to $C_{16:1}$ was obtained at 0.3 μg/spot. The highest dose tested (3 μg/spot) of $C_{16:0}$ acid gave an *AI* of 71 ± 18%. The dose-response to $C_{18:1}$ acid paralleled that of $C_{16:0}$, and the avoidance index at only one dose (0.3 μg/spot) was statistically different between the two acids (Figure 1). The difference in the avoidance index for $C_{16:0}$ and $C_{18:1}$ acids at 3 μg/spot was not significant ($Z = -1.12$, NS). $C_{16:0}$ acid induced avoidance indices higher than 80% in seven of 21 replicates, but a similar avoidance index

TABLE 1. IDENTIFICATION OF FATTY ACIDS IN METHYLENE DICHLORIDE EXTRACT OF *L. botrana* EGGS[a]

		Extraction solvent	
		Methylene dichloride	Methanol[a]
Myristic acid	$C_{14:0}$	3.91	0.26
Palmitic acid	$C_{16:0}$	30.0	1.37
Palmitoleic acid	(Z9)-$C_{16:1}$	12.5	n.f.[b]
Stearic acid	$C_{18:0}$	14.0	0.18
Oleic acid	(Z9)-$C_{18:1}$	32.5	n.f.
Linoleic acid	(Z9,Z12)-$C_{18:2}$	21.0	n.f.
Linolenic acid	(Z9,Z12,Z15)-$C_{18:3}$	15.7	n.f.
Methyl palmitate	me$C_{16:0}$	trace	1.37
Methyl palmitoleate	me(Z9)-$C_{16:1}$	trace	0.08
Methyl stearate	me$C_{18:0}$	n.f.	0.29
Methyl oleate	me(Z9)-$C_{18:1}$	trace	0.61
Methyl linoleate	me(Z9,Z12)-$C_{18:2}$	n.f.	0.23
Methyl linolenate	me(Z9,Z12,Z15)-$C_{18:3}$	n.f.	0.20

[a] Second column represents the analysis of a methanol extract published previously [Thiéry et al. (1992) produced with the permission of the publisher]. All amounts are expressed in nanograms per egg equivalent.
[b] n.f. = not found

was obtained in only two of 21 replicates with $C_{16:1}$ acid and in three of 19 replicates with $C_{18:1}$ acid.

Myristic ($C_{14:0}$), linoleic ($C_{18:2}$), and stearic ($C_{18:0}$) acids provoked little avoidance (range 6 ± 5% to 11 ± 9%) at 0.3 μg/spot, but mean indices were still different from those induced by the controls Cs or Cw (smaller difference: $Z = -3.18$, $P = 0.001$). As dose of 3 μg/spot of each of these three acids was necessary to obtain a range of avoidance similar to that induced by 0.06 μg/spot of $C_{16:0}$, $C_{16:1}$, or $C_{18:1}$ (bigger difference, $Z = -0.15$, NS).

In order to determine if esterification affected the response of moths, we tested esters of the two most effective free acids, palmitic and oleic. Methyl palmitate (me$C_{16:0}$), ethyl palmitate (et$C_{16:0}$), and methyl oleate (me$C_{18:1}$) produced very similar avoidance responses, characterized by bell-shaped dose-response curves. The dose of 1.5 μg/spot was most avoided, compared to the two other doses (smaller difference, $Z = -2.93$, $P = 0.003$).

One way to illustrate the avoidance response to each FA is to take into

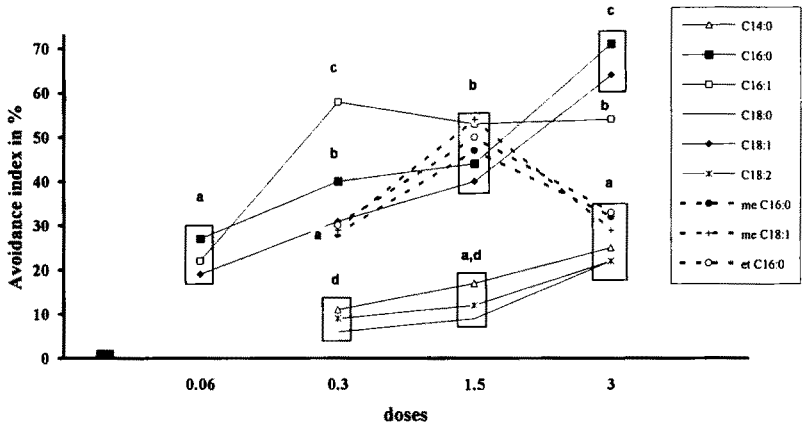

FIG. 1. Oviposition site preferences of *L. botrana* females exposed to six fatty acids and three esters. Values represent an avoidance index (AI) varying from 0% (no avoidance) to 100% (maximum avoidance). Doses are expressed as picograms/spot of application; 0.06 pg of $C_{16:0}$ is equivalent to two eggs of *L. botrana*. Black vertical bar represents AI obtained in response to the solvent alone (Cs). Values inside a frame are not statistically different at $P < 0.01$.

account its amount per egg (Table 2) and to estimate an expected number of eggs per application spot that would be necessary to produce given levels of avoidance if eggs produced a single compound. For example, 10 eggs producing only $C_{16:0}$, or between 5 and 25 producing $C_{16:1}$, would provoke an avoidance

TABLE 2. ESTIMATION OF NUMBER OF EGG EQUIVALENTS FOR EACH FATTY ACID NECESSARY TO RELEASE DIFFERENT PERCENTAGES OF AVOIDANCE (AI)

	AI (%)				
	25	40	50	60	70
$C_{14:0}$	750	no[a]			
$C_{16:0}$	<2	10	50	50–100	100
$C_{16:1}$	5	5–25	5–25	5–25	no
$C_{18:0}$	>210	no			
$C_{18:1}$	2–10	45	45–90	90	no
$C_{18:2}$	200	no			

[a]no = AI not obtained.

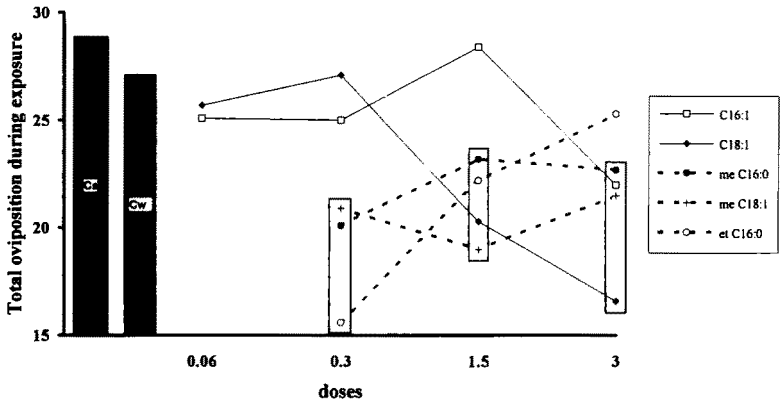

FIG. 2. Effect of the exposure to fatty acids or esters on the number of eggs laid by *L. botrana* on the whole substrate; only compounds that influenced egg laying (compared to Cs) are represented. Doses are expressed as micrograms per spot of application. Black vertical bar represents the number of eggs when exposed to the application phase and the striped bar when exposed to paraffin oil. Values inside frames are statistically different from controls (Cs and Cw) at $P < 0.05$.

index of 40%. Nearly 45 eggs producing only $C_{18:1}$ would be necessary to produce identical avoidance (Table 2).

Egg Production during Exposure. The number of eggs laid per female in Cs controls (29 ± 8.8 eggs) was very close to that obtained in Cw controls (27 ± 6.9 eggs) ($Z = -0.68$, NS). These numbers were slightly lower than the 39 ± 5.1 to 43 ± 15.6 eggs previously observed (Thiéry and Gabel, 1993), probably because of strain differences. The three esters and $C_{18:1}$ reduced the number of eggs significantly per female compared with Cs (Figure 2). The strongest reductions were obtained, respectively, with 0.3 μg/spot of $etC_{16:0}$ (15.6 ± 4.6 eggs, $Z = -3.43$, $P = 0.0006$), or $meC_{16:0}$ (18.9 ± 10 eggs, $Z = -2.86$, $P = 0.004$), and 3 μg/spot of $C_{18:1}$ (16.6 ± 12 eggs, $Z = -3.33$, $P = 0.0009$). These three values were not statistically different from each other. There was a marginal increase due to $C_{16:0}$ at each dose tested, but none was statistically different from the control (bigger difference, $Z = -1.39$, NS).

In anticipation of application of fatty acids in vineyards, we have considered the number of eggs laid per female on treated surfaces. In this we combined the avoidance response and the regulation of the number of eggs laid (Figure 3). The lowest numbers of eggs were laid on areas treated with $C_{18:1}$ at 3 μg/spot (1.1 ± 1.1 egg). About two eggs were laid on treated areas either baited with $meC_{18:1}$ at 1.5 μg/spot (1.8 ± 1.7), $C_{16:0}$ at 3 μg/spot (2.1 ± 1.8), and $C_{16:1}$ at 0.3 μg/spot (2.2 ± 1.7) (Figure 3). Ethyl palmitate, which was not found in

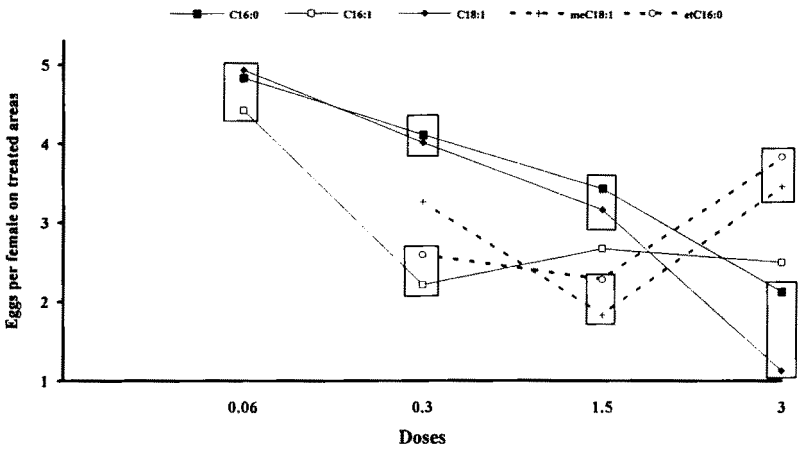

FIG. 3. Mean numbers of egg laid by *Lobesia botrana* on the treated areas (1/3 of the total area). We only considered the chemicals that reduced this amount up to ca. 2 eggs at one of the tested doses: palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$) and oleic acid ($C_{18:1}$), methyl oleate ($meC_{18:1}$) and ethyl palmitate ($etC_{16:0}$). Controls: Cs (6.8 $\pm$ 1.8 eggs) and Cw (7 $\pm$ 2.1 eggs) are note represented. Doses are expressed as micrograms per spot of application. Values inside a frame are not statistically different at $P < 0.01$.

the egg extract, also induced a low number of eggs (2.3 $\pm$ 1.1 eggs) at 1.5 μg/spot (Figure 3), but in none of these tests with esters were the numbers of eggs different ($C_{18:1}$, 3 μg, versus $C_{16:1}$, 0.3 μg: $Z = -1.27$, NS).

DISCUSSION

We have identified several fatty acids in addition to those previously reported in the eggs of *L. botrana*. We question the quantity of methyl esters previously identified. By extracting eggs in methylene dichloride, we harvested >20 times the amounts of each free fatty acid than reported previously with methanol as solvent (Thiéry et al., 1992) and only traces of two esters. We suggest that free fatty acids were underevaluated and that esters were overevaluated in our previous work due to esterification by the extraction solvent, methanol. Esterification could be obtained experimentally by dissolving FAs in cold methanol, and this will be detailed elsewhere (Farkas et al., unpublished data). The present analysis of an extract of *L. botrana* eggs revealed similar constituents as in *C. pomonella* eggs, but with different concentrations and ratios (Thiéry et al., 1995). However, occurrence of methyl esters both in methylene dichloride and ethyl ether extracts of *C. pomonella* eggs suggests that fatty acid esters are

probably produced in low amounts by eggs (Thiéry et al., 1995). The present identification is consistent with that for other tortricids such as *C. pomonella* (Thiéry et al., 1995), *Cydia molesta* and *Eupoecifia ambiguella* (P. Farkas, B. Gabel, and D. Thiéry, unpublished data).

We now propose that at least three fatty acids (palmitic, palmitoleic, and oleic) may participate in the regulation of the oviposition site selection by EGVM. In addition, two esters (methyl oleate and methyl palmitate) reduced the number of eggs laid. Ethyl palmitate was not detected in the eggs, but it reduced the number of eggs laid. The highest avoidance indices obtained with the saturated C_{16} and the monounsaturated C_{18} suggest that *L. botrana* females perceive and react to both chain length and chain unsaturations, with saturated stearic and diunsaturated linoleic acids evoking weaker responses than oleic acid. We previously reported avoidance and also reduction in the number of eggs laid by *L. botrana* in response to a blend of three fatty acids and six methyl esters of fatty acids in which palmitic acid and methyl oleate were the two major compounds (Thiéry et al., 1992; Thiéry and Gabel, 1993). Avoidance indices between 55% (Thiéry et al., 1992) and 64% (Thiéry and Gabel, 1993) were obtained using ca. 30 ng/spot of palmitic acid blended with other molecules, while 60 ng/spot of pure palmitic acid was necessary to obtain an avoidance index of only 30%. Even if palmitic, palmitoleic, and oleic acids explain most of the avoidance observed, we suspect that other fatty acids and methyl esters can synergize this effect. We also reported that a blend of three acids and six esters reduced the number of eggs laid by EGVM females (Thiéry and Gabel, 1993) and by CM females (Gabel and Thiéry, 1994). Long-chain free FAs and esters are probably commonly produced by eggs or larvae of several species; these compounds have already been found in eggs from several Lepidoptera (see references above), and larval cuticles of honeybees (Trouiller, 1993). Several works report the effect of fatty acids on egg or larval parasites. Rickli et al. (1992) demonstrated by using a servosphere apparatus that a distant source of palmitic acid improved the orientation of the bee mite *Varroa jacobsoni*. The generalist parasitoid of Lepidoptera eggs, *Trichograma brassica*, raises its locomotor activity in response to a distant source of oleic acid (Frenoy et al., 1992). These observations support the hypothesis that long-chain free fatty acids and derivatives may be involved in the perception of young instars.

Palmitoleic, oleic, and linoleic acids also deter oviposition in the mosquito *Culex quinquefasciatus* (Hwang et al., 1984), but saturated acids were not assayed in their work. The decision to oviposit or not often results from the balance between oviposition stimulants and deterrents (Renwick, 1989). In that respect chemical cues involved in the dispersion or aggregation of progeny may interact with those produced by the plant that stimulate oviposition. EGVM avoid oviposition in response to fatty acids produced by its eggs and also typically oviposit on berries that are suspected to bear lower amounts of palmitic

acid compared to grape leaves. Whether females perceive contrasts between chemical backgrounds, such as fatty acids produced by plant surfaces, or especially high amounts of fatty acids and esters corresponding to eggs needs further study.

One purpose of this work was to focus on the most oviposition deterring chemicals that can potentially be applied in the field, i.e., that are inexpensive and stable compounds. Therefore, we disregarded linolenic acid with three double bonds as too unstable. Palmitic acid and oleic acid are good candidates to explain the avoidance induced by the egg extract. Their greater stability and efficacy may make them good candidates for further field applications. The two esters are poor candidates because the number of eggs laid on treated areas increased with the dose. A definitive selection of active chemicals that could be applied in vineyards should, however, take into consideration their physical properties and their behavior under field conditions, which were not assayed in this work.

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CONTRASTING REACTIONS OF LOGGERHEAD SHRIKES TO TWO TYPES OF CHEMICALLY DEFENDED INSECT PREY[1]

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Abstract—Feeding tests with loggerhead shrikes (*Lanius ludovicianus*) showed this bird to accept *Utetheisa ornatrix*, a moth (Arctiidae) protected by pyrrolizidine alkaloids, but to avoid *Lytta polita*, a beetle (Meloidae) containing cantharidin.

Key Words—Arctiidae, Meloidae, *Utetheisa ornatrix*, *Lytta polita*, pyrrolizidine alkaloids, cantharidin, feeding aversion.

INTRODUCTION

The moth *Utetheisa ornatrix* (family Arctiidae) and the beetle *Lytta polita* (family Meloidae) are both chemically protected, the former by pyrrolizidine alkaloids (PAs) sequestered from the larval food plant (Eisner and Meinwald, 1995), the latter by cantharidin, produced by the beetle itself (McCormick and Carrel, 1987). We recently undertook some feeding tests with loggerhead shrikes (*Lanius ludovicianus*, family Laniidae), in the expectation that the birds would reject both these insects, but found that they spurned *L. polita* only, and fed readily

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on *U. ornatrix*. We here present the results of these tests and discuss their implications.

METHODS AND MATERIALS

The tests were conducted in June 1993 on the grounds of the Archbold Biological Station, Lake Placid, Highlands County, Florida, where loggerhead shrikes and both species of insect coexist naturally.

Loggerhead shrikes are sit-and-wait predators whose diet comprises mostly arthropods (Yosef and Grub, 1993), including a wide range of Lepidoptera. Like other shrikes, they commonly impale prey on sharp objects prior to consumption. Yosef and Whitman (1992) found loggerhead shrikes to impale chemically noxious prey for periods of up to three days (e.g., the lubber grasshopper, *Romalea guttata*; the danaid butterfly, *Danaus gilippus*; the southern narrow-mouthed toad, *Gastrophyne carolinensis*) and argued that such preingestive exposure of prey could provide time for spontaneous chemical breakdown of deterrent substances contained therein. However, shrikes do not invariably impale their prey. Loggerhead shrikes, for instance, at our Florida study site, were repeatedly noted in the field to capture beetles and moths and to ingest these outright (Yosef, unpublished).

U. ornatrix contain PAs at all stages of development. PAs sequestered by the larvae are retained throughout metamorphosis and eventually are transmitted in part to the eggs. Eggs, larvae, and adults are protected against arthropod predators as a result (Eisner and Meinwald, 1995). Whether PAs protect *U. ornatrix* also against vertebrate predators had not been established.

U. ornatrix can be raised in the laboratory to be either PA-containing or PA-free. The latter moths are obtained by raising larvae on a semisynthetic, pinto bean-based diet (PB diet) devoid of PA (Miller et al., 1976). PA-containing moths are obtained by raising larvae on CS diet, consisting of a blend of PB diet and seeds from one of the normal food plants (*Crotalaria spectabilis*) of *U. ornatrix* (Bogner and Eisner, 1991). The principal PA in the seeds of this plant is monocrotaline. Adult *U. ornatrix* raised on CS diet contain over 0.5 mg monocrotaline (Bogner and Eisner, 1991), an amount commensurate with the level of PA present in field-collected *U. ornatrix* (field-collected moths from our Florida site contain primarily usaramine, the principal PA in *Crotalaria mucronata*, the dominant *U. ornatrix* food plant in the area; usaramine and monocrotaline are closely similar chemically) (Conner et al., 1990; Eisner, unpublished).

U. ornatrix commonly respond to disturbance by emitting a froth from the neck region (Figure 1). The fluid, which consists mostly of blood, contains PA in both field-collected and CS-diet-raised individuals (Eisner, unpublished).

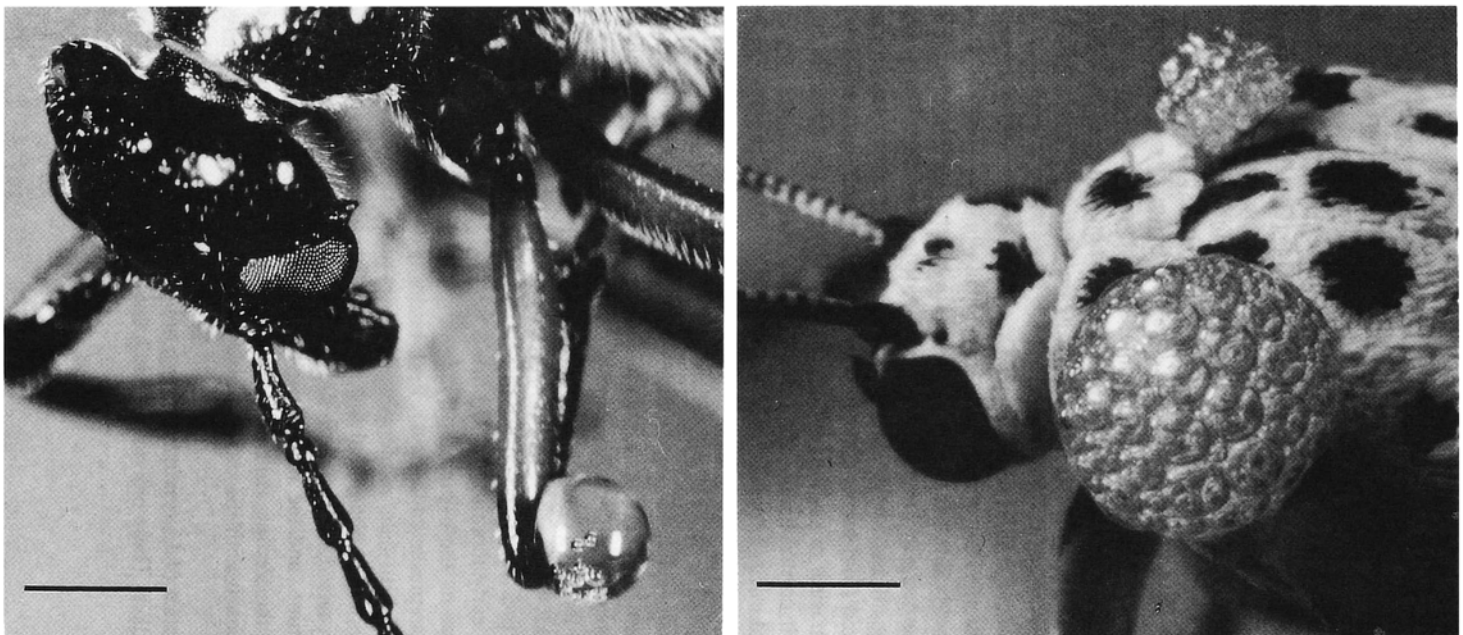

FIG. 1. *Lytta polita* (left) and *Utetheisa ornatrix* (right) responding to disturbance by emitting, respectively, a droplet of blood from a knee joint (reflex bleeding) and froth from the neck region. Bars = 2 mm (left) and 1 mm (right).

U. ornatrix, unlike moths generally, take readily to the wing in the daytime. The moth is distinctly aposematic, being white and pink with black and yellowish markings. It is therefore exposed and discernible to diurnal predators.

L. polita shows the general characteristic of Meloidae of reflex bleeding from the femorotibial joints when disturbed (Figure 1) (Carrel and Eisner, 1974). As is true for Meloidae generally, the emitted fluid contains cantharidin. Actual levels of cantharidin in the blood of *L. polita* are in the range of 10^{-1}–10^{-2} M (J. Carrel, unpublished); whole *L. polita* may contain as much as 0.4 mg cantharidin (McCormick and Carrel, 1987). *L. polita* are evenly greyish brown in coloration. While lacking the gaudy coloration characteristic of aposematic insects, they are nonetheless visually conspicuous when resting on their preferred feeding site, the male cones of slash pine (*Pinus elliottii*), from which they derive pollen (Carrel et al., 1990).

Ten loggerhead shrikes were trapped (Yosef and Lohrer, 1992) for purposes of experimentation. They were placed individually in 3-m^3 screened cages, to which they quickly adapted. Each cage bore a central upright post (1.3 m), outfitted with barbed wire around the top to provide for prey impalement. Opaque plastic sheeting affixed to the sides of the cages ensured that the birds were visually insulated from one another. Following experimentation, all birds were released at their sites of capture and were noted in the days thereafter to have returned to their normal haunts and behaviors (foraging, brooding).

Insects and mice (*Mus musculus*) that we fed to shrikes in our experiments (including to free-ranging shrikes) had all been killed shortly beforehand by freezing.

For the first days following capture, the birds were offered only innocuous prey (mice, plus various insects known or suspected to be chemically undefended). The prey were offered in deep plastic trays (12-cm-high sides, opaque plastic), which the birds came to associate with food presentation by the second day (they quickly approached the trays to investigate their holdings as soon as the trays were presented). To ensure that the birds were hungry, feedings were initiated in the early morning (07:00 hr).

Subsequent to these preliminary feedings, tests were carried out with *U. ornatrix* (also presented in trays). Each bird was offered a total of six moths, comprising one pair (male and female; the sexes are easily told apart by structural differences of the abdominal tip) of each of the three categories: PB-diet-raised, CS-diet-raised, field-collected. The moths were offered a pair of each category at a time, and the fate of each item was noted. Forty minutes after termination of the feeding sequence, each bird was offered a mouse, to check whether experience with the moths had in some fashion affected their feeding behavior.

A separate set of *U. ornatrix* presentations was done with five free-ranging loggerhead shrikes. These individuals had habituated to R.Y., who could readily

attract them to prey offered on the palm of the hand. Each of these birds was also offered six *U. ornatrix* (a male and female of each of the three categories) presented one at a time on the hand in varying order, in a single feeding session. Twenty minutes after presentation of the last moth, each bird was manually given a mouse. The tests with free-ranging shrikes were carried out at the same time as those with the caged specimens; there was therefore certainty that the two sets of birds comprised different individuals.

The experiments with *L. polita* were carried out with the trapped shrikes only and involved presentation (for a period of 6–7 hr) of mixed assortments of insect prey. These experiments were initiated some days following the tests with *U. ornatrix* and extended for four consecutive days. Observation during the daily experimental period was continuous for the first hour, and then intermittent at intervals of 2–3 hr. On day 1, each bird was given a tray bearing 20 mealworms (larvae of *Tenebrio molitor* beetles; family Tenebrionidae), plus a number of mice. On day 2, a tray was offered bearing 20 mealworms, plus 20 *Loxa flavicollis* (a stink bug, family Pentatomidae) and 20 dung beetles (*Dycenetus morato*, family Scarabaeidae). On day 3, the tray was loaded as on day 2, but contained an additional 20 longhorned beetles (*Monochamus* spp., family Cerambycidae). On day 4, the assortment equaled that of the previous day, but included an added five *L. polita*. An effort was made to keep the arrangement of insects on the trays variable.

Shrikes characteristically regurgitate residual stomach contents in the form of discrete pellets, which they void at intervals following ingestion. We found such pellets to be disgorged by our cages shrikes, and collected these pellets from the cage floors on the days of experimentation and on the following mornings prior to renewed feeding. The pellets were individually checked under the stereomicroscope for undigested remains (bones, fur, insect parts).

RESULTS

The *U. ornatrix* were all eaten, both by the caged and free-ranging shrikes. The caged birds took the moths individually in relatively quick succession (within a span of 14 min or less) and showed no hesitation in seizing or swallowing the moths. There were also no ill effects noticed in the 40 min period following the testing sequence. All birds avidly ate the individual mice that were offered to them at the end of that interval.

The free-ranging shrikes also failed to show any reluctance vis-à-vis the moth offerings. They came to the hand quickly to take each moth, and unhesitatingly ate the mouse later offered.

Scrutiny of the pellets from the caged shrikes revealed no remnants (except scales) of *U. ornatrix* (only bones and fur from the mice).

TABLE 1. NUMBER OF INSECT PREY EATEN BY TEN LOGGERHEAD SHRIKES ON DAYS 3 AND 4 OF 4-DAY EXPERIMENT[a]

Prey species	Number eaten ($X \pm$ SEM)	
	Day 3	Day 4
Mealworm, *Tenebrio molitor*	18.6 ± 0.6	18.4 ± 0.6
Pentatomid bug, *Loxa flavicollis*	18.8 ± 0.5	18.9 ± 0.4
Dung beetle, *Dycenetus morator*	18.5 ± 0.5	19.6 ± 0.2
Cerambycid beetle, *Monochamus* sp.	18.7 ± 0.4	19.7 ± 0.2
Meloid beetle, *Lytta polita*		none touched

[a]Each shrike was given 20 of each insect on each day, except of *L. polita*, which were offered, 5 per shrike, on day 4 only.

The four-day test sequence culminating in the *L. polita* presentations revealed, first, that loggerhead shrikes are voracious insectivores. The birds ate virtually all insects offered, which on days 3 and 4 amounted to nearly 80 items (Table 1). Consumption rate for these two days was calculated to average 15 ± 1.9 (SD) insects/shrike/hr. However, rate of insect intake was not constant, since the birds paused at intervals during the feeding sessions for relatively lengthy periods prior to ejection of individual gastric pellets (during the first hour of prey presentation, we timed such pauses to be in the range of 27–48 min). A total of 56 pellets were produced by the shrikes during the four days of experimentation. Examination of these pellets revealed ample skeletal parts from the beetles and pentatomids. None of these insects was regurgitated intact with the pellets.

The salient finding from the four-day test was that the *L. polita* offered on day 4 were all ignored. In not a single instance was one of these beetles noted to be taken in the bill by a bird. The rejections were all effected on the basis of noncontact assessment.

The feeding behavior of the shrikes in the four-day test was quite consistent. Typically, birds hopped into the tray and remained in it for 3 sec or less as they chose and consumed an individual insect. Two of the birds differed somewhat from the others in that they tended first to drop the prey on the ground beside the tray and to retreat briefly to a perch, before returning to the item to eat it. In not a single instance in our tests with caged shrikes was an insect impaled on the barbed wire of the pole. Mice were always impaled by the shrikes, to facilitate the butchering process.

DISCUSSION

U. ornatrix and *L. polita* were evidently treated very differently by our shrikes. The birds ate all 90 *U. ornatrix* offered, without hesitation and without betraying ill-effects. We are reluctant to conclude, however, that loggerhead shrikes fail to taste PAs or that they are fully tolerant of the chemicals. PAs are strongly toxic to vertebrates (hepato-, pneumo-, and nucleotoxic), and the toxicity is sometimes of delayed onset (Mattocks, 1972; Hartman, 1991). A more lengthy series of *U. ornatrix* feedings (our birds ate only four PA-laden moths, all on the same day, and may have received no more than 2–3 mg PA each) might thus well have had deleterious effects on the birds and led these eventually to discriminate against the moths on the basis of taste or sight. We were reluctant to expose the shrikes to potential risk by feeding them *U. ornatrix* in greater numbers. Shrikes are on the decline worldwide, a fate they are not escaping in Florida (Yosef et al., 1993). One point worth noting is that loggerhead shrikes do appear to consume *U. ornatrix*, at least at times, in nature. We have noted occasional specimens of the moth impaled at actual prey caches of the birds in Florida (Yosef, unpublished).

The fact that no trace (except scales) of *U. ornatrix* could be found in the pellets is not surprising, since the moth is soft-bodied and devoid of the sort of jagged, potentially abrasive parts that are usually regurgitated by shrikes with their pellets. We therefore assume all our *U. ornatrix* to have been passed without residue into the intestine of the birds.

The dead *U. ornatrix* used in our tests were obviously not able to emit froth. While this might have somewhat reduced their state of defendedness, the froth is not the only means by which *U. ornatrix* externalize their PA. The entire integument of the moth, including the wing surfaces and perhaps even the scales, is laden with PA (Eisner, unpublished). The PA in the moths should therefore have been detectable to the shrikes.

One should not assume, from our results with shrikes, that *U. ornatrix* is generally acceptable to avian predators. Preliminary data obtained by one of us (T.E.) with free-ranging scrub jays (*Aphelocoma coerulescens*; family Corvidae), indicate that these birds tend to reject the *U. ornatrix*, both on sight and after tasting. Moreover, scrub jays showed these tendencies vis-à-vis both PA-laden and PA-free (that is, PB-diet-raised) moths, indicating that compounds other than PAs may also contribute to the defense of *U. ornatrix*. It is doubtful, however, that scrub jays are major natural enemies of *U. ornatrix*, since the birds tend not to forage in the habitats frequentated by the moth.

Data on the deterrent potential of PAs to vertebrates are scant. Our findings with shrikes do not rule out the possibility that PAs might be deterrent to other birds, or for that matter to nonavian vertebrates, on account of either bitterness

or toxicity. Interestingly, PAs appear not to be effective as taste deterrents to mice (Glendinning et al., 1990).

Whether the avoidance of *L. polita* by our shrikes was a reflection of innate aversion or of discrimination based on direct experience with the beetles remains unknown. Either way, one is tempted to attribute the defendedness of the beetle to the one deterrent compound, cantharidin, that it is known to contain, but such attribution should be advanced with caution. Cantharidin is a potent feeding deterrent to insects (Carrel and Eisner, 1974) and is highly toxic to some vertebrates (Kaiser and Michl, 1958), but its actual effect on birds has yet to be properly assessed. Again, because of the potential risk to the shrikes, we were reluctant to subject our birds to feeding experiments with cantharidin itself. Interestingly, some birds are able to tolerate cantharidin ingestion (Carrel, 1971). In our judgment, one cannot rule out the possibility that compounds other than cantharidin also play a role in bird aversion to Meloidae.

Meloid beetles constitute a diverse group at our Florida site. They range broadly in size and color pattern (*L. polita* is the largest; many species are colorful) and most lead a relatively exposed adult existence. It would be interesting to know whether loggerhead shrikes discriminate against them all. We have never seen meloid beetles among the insects impaled at loggerhead shrike caches (Yosef, unpublished).

A point of interest concerns the evident acceptability of the *Loxa flavicollis* that we offered. When live, these insects, like other Pentatomidae, would have been able to spray a carbonyl compound-laden secretion (Blum, 1981) when attacked. While these insects might well have been treated differently had they been given live to our birds, the shrike must in all likelihood have gotten "a taste" of the exocrines, since remnants of the chemicals must have been present in the glands of the corpses.

The fact that the pellets produced by our shrike contained cuticular remains only when hard-bodied insects were fed (*L. flavicollis* and the various beetles), but none when a soft-bodied insect such as *U. ornatrix* was fed, indicates that pellet analysis can yield at best only partial information about dietary intake in shrikes. Pellet analyses undertaken in conjunction with studies of shrike population declines, and intended to reveal the causes of such declines, should therefore be interpreted with caution.

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INFLUENCE OF SNAIL FECES AND MUCUS ON OVIPOSITION AND LARVAL BEHAVIOR OF *Pherbellia cinerella* (DIPTERA: SCIOMYZIDAE)

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Abstract—Larvae of the sciomyzid fly *Pherbellia cinerella* are voracious predators of terrestrial helicid snails. Eggs are deposited in areas where snails occur and larvae hunt actively for their prey. Snail feces and mucus were tested to determine if they had any kairomone or stimulatory effects on *P. cinerella*. Adult flies oviposited more frequently on substrates containing fresh snail feces than on substrates containing snail mucus or water (control). However, mucus and feces both stimulated increased search behaviour in first instar larvae. These results are discussed in relation to snail biology, and the potential for augmentation of these flies in areas affected by pest snails.

Key Words—Oviposition stimulant, kairomone, searching behaviour, Sciomyzidae, snails, Mollusca, biological control.

INTRODUCTION

The family Sciomyzidae are parasitoids and predators of both terrestrial and aquatic snails. Since Berg (1953, 1966) drew attention to the malacophagous nature of sciomyzids and their potential as biological control agents of medically important snails, intensive study has been carried out on their biology (see review in Berg and Knutson, 1978). Attempts at using these flies as biological control agents have been aimed at the control of aquatic or semiaquatic vector snails. In Hawaii, *Sepedon macropus* Walker was released for the control of *Lymnea ollula* Gould and *Pseudosuccinea columella* Say, intermediate hosts of *Fasciola gigantica* Cobbold, a liver fluke of cattle (Chock et al., 1961). Recently, an attempt has been made to augment populations of *Sepedon scapularis* Adams

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for the control of intermediate hosts of schistosomiasis in South Africa (Maharaj et al., 1992).

Although there have been no releases of sciomyzids for the control of pest and vector terrestrial mollusks, their potential use was discussed by Berg and Knutson (1978). Knutson et al. (1970) examined the biosystematics and potential of *Salticella fasciata* Meigen as a biocontrol agent of pest helicid snails and concluded that it may have good potential due to its host specificity. Recently, the effectiveness and potential of *S. fasciata* as a biological control agent of the helicid pests *Theba pisana* Müller and *Cermuella virgata* da Costa was reexamined by Coupland et al. (1994), who concluded that the species was an inappropriate biocontrol agent due to its inability to kill the pest target snails. However, another sciomyzid, *Pherbellia cinerella* Fallén, is known to be a predator of helicid snails, but its potential as a biological control agent has never been tested.

Most Diptera oviposit on or near their food source. Some Sciomyzidae, such as *S. fasciata*, lay their eggs directly on the host snail at the umbilicus (Knutson et al., 1970). In other species, such as *P. cinerella*, eggs are laid on the ground and first-instar larvae actively search for prey snails. Host-produced chemicals that stimulate both increased oviposition and search behavior have been documented for several dipteran predators and parasites of Lepidoptera and earthworms (Morris and Pivnick, 1991; Nettles and Burke, 1975). Snail mucus is known to induce trail following in several insect predators of snails such as the Lampyridae (Schwalb, 1961). In addition, several species of snails use mucus in homing, food finding (especially carnivorous snails), and courtship behavior (Cook, 1992). Snail feces have been implicated in interference competition between *T. pisana* and *C. virgata*. As part of a general study on the biology of *P. cinerella* and its suitability as a biological control agent of pest helicid snails, this paper reports on the oviposition and first-instar larval responses of *P. cinerella* to feces and mucus of the snail *C. virgata*.

METHODS AND MATERIALS

Rearing Procedure. A laboratory culture of *P. cinerella* was established using eggs of field-collected females (Viols le Fort, Herault, France). Flies were placed in male–female pairs in clear plastic rearing cages (16 × 14 × 14 cm) with moistened filter paper (to maintain humidity) and a stick for perching. Flies were fed a mixture of honey, brewer's yeast, and skim-milk powder and kept at 20°C under natural light conditions. Eggs were collected from rearing cages, placed on moist filter paper, and kept in Petri dishes at 21°C. After hatching, larvae were maintained with excess *C. virgata* (approx. 5 mm in diameter).

Effect of Feces and Mucus on Oviposition. The effect of feces and mucus on the selection of oviposition site by female flies was tested using a three-

choice assay. Mated first-generation females (F_1) were introduced singly into rearing cages that contained a choice of three substrates—moist peat with feces, moist peat with snail mucus, and as a control moist peat with water—placed in small Petri dishes (5 cm diam.) on the bottom of the cage. Fresh feces from several snails (*C. virgata*) reared on carrot and oatmeal were collected from snail-rearing boxes. Approximately 0.5 g of feces was mixed thoroughly with 20 g of sterilized peat moistened with sterilized deionized water. Snail mucus was collected (approx. 5 ml) from several snails stressed by pressing on their foot with a small needle. This mucus was mixed with sterilized deionized water and the mixture was then used to moisten 20 g of peat. The control was peat moistened with the sterilized deionized water. The experiment was replicated 12 times with different flies. The eggs of *P. cinerella* laid within each dish were counted daily, fresh substrate was added, and the Petri dishes containing each substrate were replaced in random positions. The experiment continued two weeks.

Larval Behavior Experiments. First-instar larvae of *P. cinerella* crawl actively in search of host snails. During this period they often step and tap their head on the soil surface from side to side. The frequency of this tapping behavior per unit time was used to determine if feces and mucus had a stimulatory effect on search behavior. Experiments were conducted in Petri dish arenas (15 cm diam.) on filter paper. Larva were introduced and allowed to acclimatize for 1 hr on moist filter paper, after which a 10-cm-diameter disk of filter paper impregnated with either a deionized water and snail feces mixture (20 ml and 0.5 g), a deionized water and snail mucus mixture (20:5 ml ratio), or just deionized water was introduced. Care was taken not to cover the larva with the introduced disks. Introductions were done when the larva was at the side of the Petri dish. Preliminary observations indicated no behavioral differences when larvae were in different areas within the dish. Five minutes before introduction of the test disk the number of larval head taps per 5 mins was counted. When the larvae crawled on to the test disk, the number of head taps was again recorded for a period of 5 mins. This procedure was replicated 20 times for each test disk using a different larva each time. The difference between the number of taps before and during and treatment was then recorded.

Statistical Analysis. The total number of eggs laid on each substrate and the differences in number of larval taps for each treatment were compared using Kruskal-Wallis ANOVA.

RESULTS AND DISCUSSION

P. cinerella laid more eggs on the substrate that contained feces than on the substrates containing either mucus or deionized water (control) (Figure 1). The latter two were not significantly different. Larval head tapping increased in

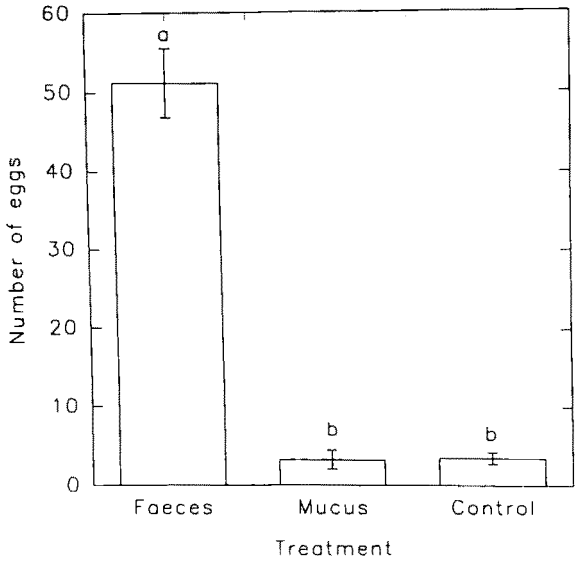

FIG. 1. Mean (±SE) number of eggs laid on each of the treatment substrates over 14 days from 12 replicates. Bars with the same letter are not significantly different, Kruskal-Wallis ANOVA, $P < 0.05$.

the presence of both feces and snail mucus, which did not significantly differ (Figure 2). The behavior of larvae presented with the deionized water (control) disks did not significantly differ from the pretreatment behavior.

While *Pherbellia cinerella* will oviposit in the absence of snail feces, as shown in this study, their presence increases the likelihood of oviposition. This is the first demonstration of a snail-produced kairomone in a snail-sciomyzid interaction. The use of only snail feces as an oviposition kairomone is an interesting result. While mucus is known to elicit a response from invertebrate predators of snails (Schwalb, 1961) and other snails (Cook, 1992), feces have never been implicated as eliciting a response. Fecal matter was shown to reduce activity in coexisting helicid snails and has been proposed as a mechanism for interspecific competition through negative effects from its ingestion. In herbivorous insects, host plant volatiles in the feces are known to act as kairomones for both their parasites (Takabayashi and Takahashi, 1989) and predators (Wyatt et al., 1993). However, since *C. virgata* is primarily a detrivore, no specific secondary plant compound would be consistently associated with its feces. One possibility may be that the kairomone is produced by gut microorganisms. These have recently been implicated in kairomone production in the leek moth (*Acrolepiopsis assectella* Zeller). In another helicid snail, *Helix pomatia* L, the bacterial

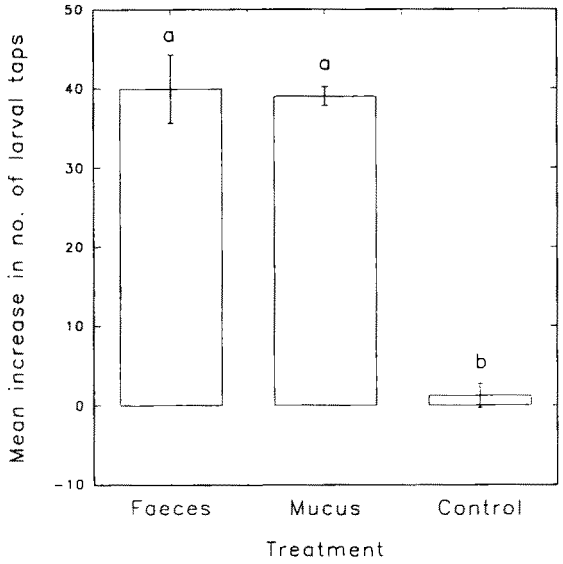

FIG. 2. Mean (±SE) increase in number of larval head taps when presented with different treatment filter paper disks. Bars with the same letter are not significantly different. Kruskal-Wallis ANOVA, $P < 0.05$.

flora were found to be the same among individuals, especially in relation to the chitinase-producing bacteria (Florkin and Lozet, 1949). It may be that the gut flora of individual *C. virgata* are also similar in species composition.

A possible reason for a predator using a fecal kairomone may be that many terrestrial mollusks, including slugs, tend to return to the same resting spot during periods of inactivity (i.e., during the day) (Cook, 1979; Lorvelec, 1990). At these resting sites, snails tend to defecate before forming a resting epiphragm (Coupland unpublished data). Therefore the presence of feces would be a good indicator that snails were in proximity. In contrast, mucus, widely spread during the previous evening of foraging, would indicate only that snails are present, with no clue as to their proximity. In contrast to *P. cinerella*, the earthworm predatory fly, *Coenesia tigrina* Fallen is stimulated to extend its ovipositor by earthworm mucus, but tests were not conducted on earthworm casts (Morris and Pivnick, 1991). Since earthworms are mainly subterranean, with only periodic activity at the surface when producing soil casts, mucus would seem to be as good as indicator of earthworm presence as their casts. However, casts have been found to be highly variable in their composition, especially with respect to microorganisms (Parle, 1963), possibly making it more difficult for a suitable component to be utilized as a kairomone.

While snail mucus had no effect on the oviposition behavior of *P. cinerella*, it stimulated increased searching (head tapping) behavior in first instars. Feces had a similar effect, although this may be due to contamination as feces are inevitably contaminated with some mucus. Snail mucus is also used by other invertebrate snail predators, notably *Lampyris* spp. (glow-worms), that follow mucus trails. Glow-worm larvae are so sensitive to mucus that they can distinguish between mucus secreted at the head and tail end of a snail by its chemical composition. *Lampyris* spp. use this information to inject poison into the anterior end of the snail, paralyzing it more quickly (Schwalb, 1961).

Pherbellia cinerella is a very common pasture-inhabiting fly that attacks and kills a wide range of snail species (Coupland and Baker, 1995). In most pastures in the south of France there are two to seven snail species that coexist, all of which are attacked by *P. cinerella*. Furthermore, many of these species are pests of both pasture and crops in Australia and in regions of the Mediterranean (Baker, 1986). If a kairomone could be identified and manufactured, it may be possible to use it to augment or direct populations of adult *P. cinerella* in or to areas where such snails are a problem.

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BEHAVIORAL OBSERVATIONS AND MEASUREMENTS OF AERIAL PHEROMONE IN A MATING DISRUPTION TRIAL AGAINST PEA MOTH *Cydia nigricana* F. (LEPIDOPTERA, TORTRICIDAE)

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Abstract—Synthetic sex pheromone of the pea moth *Cydia nigricana*, (*E*,*E*)-8,10-dodecadien-1-yl acetate (*E*8,*E*10-12 : Ac), was applied in a 3-ha pea field at a rate of 17 g/ha, in two different dispenser formulations. Aerial concentrations within pea canopy, as determined by a field electroantennogram (EAG) apparatus, were 2 and 3 ng/m^3 in the two dispenser treatments. The validity of the EAG measurements was corroborated by sampling of field air, followed by gas chromatographic quantification of *E*8,*E*10-12 : Ac. Males were attracted to fresh dispensers releasing *E*8,*E*10-12 : Ac plus less than 2% of the antagonistic *E*, *Z*; *Z*, *E*; and *Z*, *Z* isomers. Two days after placement, the proportion of these isomers had increased to 6%. Males were then no longer attracted to the dispensers, but were observed to fly out of the treated field. Male attraction to calling females was almost entirely suppressed, and attraction to traps baited with synthetic pheromone was significantly reduced. Larval infestation in the pheromone-treated field was 2%, compared to 36% in a control field.

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Key Words—Sex pheromone, (*E*,*E*)-8,10-dodecadien-1-ol acetate, antagonist, inhibitor, mating disruption, behavioral observations, field EAG, *Cydia nigricana*, Tortricidae, Lepidoptera

INTRODUCTION

The potential of a blend of sex pheromone (*E*,*E*)-8,10-dodecadien-1-yl acetate (*E*8,*E*10-12 : Ac) and the antagonistic isomers *E*,*Z*-, *Z*,*E*-, and *Z*8,*Z*10-12 : Ac (Witzgall et al., 1993) for mating disruption of the pea moth, *Cydia nigricana* F., has recently been demonstrated. After isomerization of initially pure *E*8,*E*10-12 : Ac in the dispensers, *C. nigricana* males were repelled from a 0.6-ha pea field, and male attraction to traps baited with *E*8,*E*10-12 : Ac was suppressed during the test period of 35 days (Bengtsson et al., 1994). However, larval infestation in the treated field was not significantly different from the adjacent control field. For the present study, a larger and isolated field site has been chosen to minimize immigration of mated females.

Although males were no longer seen in the treated field a few days after dispenser placement (Bengtsson et al., 1994), short-range attraction of males to females within the canopy might have been overlooked. It therefore remained to be determined whether traps with synthetic *E*8,*E*10-12 : Ac on rubber septa were indeed suitable to monitor disruption of sexual communication in *C. nigricana*. Although traps and septa were exchanged regularly, they may have been less attractive than females, due to isomerization of *E*8,*E*10-12 : Ac (Witzgall et al., 1993). During the present study, we have observed male attraction to calling females, and a propheromone compound, releasing pure *E*,*E* isomer (Streinz et al., 1993).

The measurement of the dispersal and aerial concentrations of mating disruptant chemicals within the crop is fundamental to the optimization of dispenser load and placement and the comparison of different experiments. A portable electroantennogram (EAG) apparatus, using the male antenna as sensor, allows direct detection of ambient pheromone (Sauer et al., 1992). For determination of absolute concentrations in field air, the antennal response was correlated with stimulus concentrations in syringes (Bengtsson et al., 1994). To substantiate this calibration, we have now concurrently determined aerial pheromone concentrations by field EAG and by gas chromatographic analysis of air samples (Caro et al., 1978, 1979).

METHODS AND MATERIALS

Chemicals and Dispensers. *E*8,*E*10-12 : Ac was formulated in puzzlepiece configuration trilaminate dispensers (Hercon Environmental Co., Emigsville,

Pennsylvania) and in polyethylene tube dispensers (rope dispensers) (Shin-Etsu Chemical Co., Tokyo), at 50 mg *E*8,*E*10–12:Ac/dispenser. Isomeric purity of *E*8,*E*10–12:Ac before formulation was 99.6% and 99.1%; chemical purity was 99.7% and 99.7% by capillary gas chromatography (GC). Rope dispensers from the same batch had been used in a preceding study (Bengtsson et al., 1994).

For field trapping (see below), we used HPLC-purified *E*8,*E*10–12:Ac (99.8% isomeric purity by GC) and a propheromone compound, tricarbonyl [(8,9,10,11-η)-8,10-dodecadien-1-yl acetate] iron, releasing isomerically pure E8,E10–12:Ac (Streinz et al., 1993).

Gas Chromatography. GC analyses were done on Hewlett Packard 5890 instruments with flame ionization detection, on a DB-Wax, and on a DB-1 column (J&W Scientific, Folsom, California) 30 m × 0.25 mm ID. The temperature was programmed from 60°C at 10°/min to 100°C, 1.5°/min to 150°C, and 20°/min to 230°C.

Measurement of Release Rates. Release of Δ8, Δ10–12:Ac from the dispensers, rubber septa, and propheromone filter papers was measured in 250-ml round-bottom flasks in a static atmosphere. The dispensers were suspended for 2 hr, and the atmospheric concentration in these flasks was saturated after >5 hr (see Bengtsson et al., 1994). Dispensers were collected from the field nine times, 0–71 days after application, and were stored at −20°C until analysis. Rubber septa and propheromone papers were aged in the laboratory at 25°C.

Release of *E*8,*E*10–12:Ac from individual *C. nigricana* females was measured during the first 30 min of calling. A constant airstream (100 ml/min) passed over the female in a cylindrical holding chamber (1.5 × 3 cm long), into glass capillary tubes (0.1 × 12 cm long) (Witzgall and Frérot, 1989). The capillaries were eluted with 5 μl of redistilled heptane.

Field Facilities and Dispenser Placement. The test field (60 × 500 m) at Veberöd (Skåne, Sweden, 1993) was 8 km from the nearest pea field. The long dimension was perpendicular to the main wind direction; the downwind side was bordered by trees and shrubs, and the upwind side was adjacent to vegetable cultures and open meadows. In 1992, 3 ha of peas had been grown at 200 m upwind; this field had been infested with pea moths (Table 1). The test field and a control field (4 ha at Höör, 30 km from Veberöd), were sown with green peas, *Pisum sativum* L., of the variety Capella.

The test field was partitioned into a grid of adjacent 10- × 10-m squares. Dispensers were attached to top shoots of pea plants on the flagged corners and on the middle of the sides of each square, on June 12, 1993, several days before the first peas were flowering. Dispenser density was 8 per "unit square"; overall density was 333 dispensers/ha (16.5 g *E*8,*E*10–12:Ac/ha). A total of 880 laminate dispensers was placed on 60 × 440 m and 120 rope dispensers on 60 × 60 m. In addition, eight laminate or eight rope dispensers were placed in two single 100-m^2 squares within the control field.

TABLE 1. MATING DISRUPTION OF *C. nigricana* IN 3-HA PEA FIELD (VEBERÖD, 1993)[a]

	Dispenser material	
	Trilaminate	Polyethylene tube
Area treated	0.6 ha of 3-ha pea field	2.4 ha of 3-ha pea field
Active ingredient	*E*8,*E*10-12:Ac (50 mg/dispenser)	
Amount applied	17 g/ha (330 disp/ha)	
Release rate (10–34 days)[b]	1.7 ± 1.5 μg/disp/hr	5.7 ± 2.2 μg/disp/hr
	14 mg/ha/day	45 mg/ha/day
Blend composition (10–34 days)	16 ± 9% *E*,*Z*-, *Z*,*E*-,*Z*8,*Z*10-12:Ac	8 ± 2% *E*,*Z*-,*Z*,*E*-,*Z*8,*Z*10-12:Ac
Activity of nonpheromonal isomers	Attraction antagonists	
Aerial concentration of *E*8,*E*10-12:Ac within canopy, by		
Field-EAG (21–36 days)	1.6 ± 1.2 ng/m^3	2.7 ± 1.9 ng/m^3
Air sampling (21–36 days)	not detected (< 1 ng/m^3)	1.3 ± 0.5 ng/m^3
Communication disruption (Figure 2)		
Calling females (9–37 days)	98% reduction	100% reduction
Traps (100 μg *E*8,*E*10-12:Ac) (0–65 days)	64%	96%
Traps (3 mg propheromone) (0–65 days)	68%	87%
Larval infestation	larvae/m^2	% infested pods
Control field		
Höör, 1992	67 ± 17	44 ± 14
Höör, 1993	48 ± 13	36 ± 16
Test field		
Veberöd, 1992	52 ± 13	34 ± 8
Veberöd, 1993	6 ± 3	2 ± 1

[a] Pea moth, *Cydia nigricana*, F. (Lep., Tortricidae); on green peas, *Pisum sativum* L. Sex pheromone: *E*8,*E*10-12:Ac (Greenway, 1984; Witzgall et al., 1993). Release: calling female, 3.1 ± 1.4 ng/hr; rubber septum (100 μg; 3 days), 18.0 ± 5.7 ng/hr; propheromone (3 mg; 1–21 days), 311 ± 38 ng/hr.
[b] Days after dispenser placement; measurement in static atmosphere.

Field Electroantennogram Recordings. The field EAG apparatus (Sauer et al., 1992) was operated according to Bengtsson et al. (1994). For measurements of ambient pheromone, air was drawn at 42 ml/sec through a glass chamber containing the antenna holder. The airstream was filtered with active charcoal for recordings of the resting potential of the antenna. The EAG response from antennae of *C. nigricana* males (1 day old) was normalized to 2-ml pulses from a syringe holding a red rubber septum with 10 μg *E*8,*E*10–12 : Ac to obtain "relative EAG amplitudes." Data were sampled at 18.2 Hz and peak heights were averaged off-line for the first 2 sec of each recording.

Each recording included three pulses of synthetic pheromone before and after each of three measurements of field air, during a period of ca. 15 min. Recordings were made at two locations in the control field (Höör) and at six locations within the test field, at $\geq$15 m downwind from the field border (Veberöd), 21–36 days after dispenser application. The apparatus was placed in the middle of 100-m^2 squares (see above) and air was drawn from 10–20 cm below canopy top.

Relative EAG amplitudes to pulses from syringes holding filter papers with synthetic *E*8,*E*10–12 : Ac had been correlated with the concentrations of *E*8,*E*10–12 : Ac in these syringes (see Bengtsson et al., 1994). Using this correlation, we calculated concentrations of *E*8,*E*10–12 : Ac in field air from the EAG amplitudes.

Air Sampling. Air from the test field was drawn by a suction pump (Brey GmbH, Memmingen, Germany) at 17 liters/min during 4 hr through a Tenax filter to adsorb ambient *E*8,*E*10–12 : Ac. The flow rate was regulated with a flowmeter (Kytölä Instruments, Muurame, Finland). The filter inlet was held at 10–20 cm below the pea canopy top. Sampling was done simultaneously with the EAG recordings at three locations in the test and control field.

The filters were glass tubes (0.5 $\times$ 4.5 cm) containing 50 mg of Tenax (ca. 0.5 cm deep), held by plugs of glass wool. They were extracted ca. 1 hr after sampling with 0.5 ml of diethyl ether (Prolabo, Paris) containing 12 : Ac as internal standard. The solvent evaporated from glass vials with an elongated tip (0.25 $\times$ 3.5 cm long) at room temperature to ca. 10 μl, 6 μl of which was injected on a DB-1 column. The remaining solvent volume was measured by a syringe and was injected on a DB-Wax column.

The outlet from the DB-1 column was split between a flame ionization (FID) and an electroantennogram detector (EAD) (Arn et al., 1975), using a male antenna of *C. splendana* for detection of *E*8,*E*10–12 : Ac (Witzgall et al., unpublished data). For quantification, area counts at the retention times of *E*8,*E*10–12 : Ac were averaged from runs on the DB-1 and the DB-Wax columns. Data were corrected for a recovery efficiency of 47%, which was determined by applying *E*8,*E*10–12 : Ac to the filters and drawing air at 17 liters/min during 4 hr.

Field Trapping. Tetra traps (Arn et al., 1979) were baited with 100 μg *E*8,*E*10–12 : Ac (99.8% isomeric purity) on red rubber septa (Thomas Scientific) or 3 mg of propheromone (tricarbonyl [(8,9,10,11-η)-8,10-dodecadien-1-yl acetate] iron) on 2.5-cm^2 filter papers (see above). Propheromone sources release isomerically pure *E*8,*E*10–12 : Ac (Streinz et al., 1993) and were not replaced during the study. Since *E*8,*E*10–12 : Ac isomerizes rapidly on rubber septa (Davis et al., 1984; Witzgall et al., 1993), traps and septa were replaced every three to five days.

Three trap pairs (*E*8,*E*10–12 : Ac and propheromone, spaced at 10 m, perpendicular to the main wind direction) were placed in both treatments within the test field and in the control pea field at >5 m from the border, on an area of 60 × 60 m. In the test field, traps were placed in the center of 100-m^2 squares, surrounded by eight dispensers (see above).

Behavioral Observations. Last instars of *C. nigricana* were field-collected at Höör in 1992 and were overwintered under field conditions. Ten females, or 10 females plus 12 males, were transferred on the day of emergence to transparent PVC cylinders (12 × 20 cm) with a white polyethylene mesh covering both openings and supplied with sucrose solution. In the test field, cages with females, cages with females and males (at 1 m distance from each other and 2–4 days old), and propheromone traps without sticky bottoms were installed in the centers of 100-m^2 unit squares at 5–7 m distance from the surrounding dispensers.

Nine to 37 days after dispenser application, insect cages and propheromone traps were observed simultaneously by two people during the diel flight period of *C. nigricana* between 4 and 9 PM. Observations in the field treatments and in the control field were done on consecutive days. The protocol included the number of upwind flights over at least 1 m distance to traps or females, followed by landing. Repeated short-range flights and landings were not recorded, and males reorienting to the female cage from several meters' distance were not distinguished from newly arriving males. The duration of female calling (abdomen lifted from support, pheromone gland visibly protruded) and matings in the cages with naive females and wild males (during three consecutive days) was recorded at 15-min intervals.

Larval Counts. The larval density in pea fields at Veberöd and Höör was monitored in 1992 (Bengtsson et al., 1994) and 1993. During August 1993, after the flight period of *C. nigricana*, squares of 1 m^2 in the test field (N = 24) and the control field (N = 20) were chosen at random and all pea pods were checked for larval damage.

RESULTS AND DISCUSSION

The principal parameters of this second mating disruption trial against pea moth, *C. nigricana*, are summarized in Table 1.

Release Rates of Disruptant Chemicals and Pheromone

Release from Dispensers. The release of *E*8,*E*10–12:Ac from the rope dispensers was 17.5 ± 5.2 μg/dispenser/hr ($N = 3$) on the first day and was then rather uniform between 2 and 71 days after placement (6.5 ± 1.7 μg/dispenser/hr; $N = 21$) (see also Bengtsson et al., 1994). The trilaminate dispensers emitted 4.3 ± 1.3 μg/hr ($N = 3$) on the first day, 3.3 ± 0.9 μg/hr between days 2 and 6 ($N = 9$), and leveled off to 0.2 ± 0.2 μg/hr between days 26 and 34 ($N = 15$) (see also Table 1). The release rates were measured in a static atmosphere and are expected to be higher in the field.

Isomeric purity of *E*8,*E*10–12:Ac was >99% before formulation. Fresh rope and trilaminate dispensers released 1.7% and 2.1% of the other isomers, *E*,*Z*-, *Z*,*E*-, and *Z*8,*Z*10–12:Ac. In the rope dispensers, the isomer proportion increased within two days to 6.4 ± 1.2% ($N = 3$) and was quite consistent over the following 69 days (8.8 ± 2.1%; $N = 21$). By comparison, in the trilaminates, the isomer proportion steadily increased from 5.6 ± 2.3% on day 2 ($N = 3$) to 34.2 ± 5.9% on day 71 ($N = 3$) (see also Table 1).

Pheromone Release from Females and Trap Baits. Female pea moths produce pure *E*8,*E*10–12:Ac. The other isomers are strong attraction antagonists (Witzgall et al., 1993). The mean release rate of *E*8,*E*10–12:Ac from individual females during the first 30 min of calling was 3.1 ± 1.4 ng/female/hr ($N = 4$). The release rate of *E*8,*E*10–12:Ac from propheromone sources was 311 ± 38 ng/hr during the first 21 days, 65 ± 48 ng/hr on day 29, and 22.5 ± 10 ng/hr on day 36 ($N = 3$). Other isomers were not detected. Traps with rubber septa were exchanged every three to five days, and the release from 3-day-old rubber septa was 18.0 ± 5.7 ng/hr ($N = 3$) (Table 1).

Dispersal and Aerial Concentrations of Disruptant Chemicals

Field EAG Recordings. Of the test pea field, 2.6 ha were treated with laminate dispensers and 0.4 ha with rope dispensers for comparison with a prior study (Bengtsson et al., 1994). EAG recordings at three locations within each treatment 21 days after dispenser placement indicated a homogenous distribution of disruptant chemical within the pea canopy. The average relative EAG amplitudes were 0.33 ± 0.09 ($N = 9$) in the laminate dispenser section and 0.54 ± 0.12 ($N = 9$) in the rope dispenser section. EAG recordings were then repeated at one location within each treatment at 26, 31, and 36 days after dispenser placement. The relative EAG amplitudes were consistently higher among rope dispensers (0.58 ± 0.18; $N = 27$) compared to laminate dispensers (0.31 ± 0.12; $N = 27$).

In the control pea field, two 100-m^2 plots were treated with eight trilaminate or eight rope dispensers. The relative EAG amplitudes in the centers of these isolated plots did not differ significantly from amplitudes recorded in the field treatments with the two dispenser types (Figure 1, A and B).

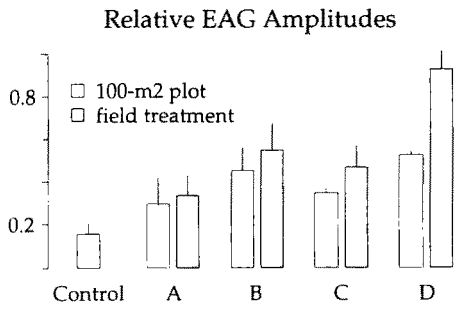

FIG. 1. Male *C. nigricana* field EAG response within pea canopy, in the center of single 100-m^2 plots in control fields (open bars) and in field treatments (shaded bars). All recordings were done in the middle of the field, except for one recording at the wind-protected border of a field treatment (D; shaded bar). A: trilaminate dispensers at 8 dispensers/100 m^2; B and C: rope dispensers at 8 dispensers/100 m^2; D: rope dispensers at 12 dispensers/100 m^2. A and B: this study. C and D: data from Bengtsson et al. (1994).

In the prior study by Bengtsson et al. (1994), the EAG apparatus was set in the center of 10- × 10-m unit squares, surrounded by 8 dispensers/100 m^2 in the field middle, and by 12 dispensers/100 m^2 close to the field border. The EAG amplitudes in the middle of this field treatment were not significantly different from a single eight-dispenser plot within the untreated control field (Figure 1, C). However, the border of the treated field was protected from wind by dense bushes, and at 5 m from the border relative EAG-amplitudes were almost twice as high as in a single 12-dispenser-plot in the open control field (Figure 1, D).

Dispersal of Disruptant Chemicals. The comparison of recordings in isolated plots and in field treatments shows that wind transport of disruptant chemical is limited within the canopy and that ambient concentrations result from a continuous release from proximate dispensers. Losses of disruptant chemical are due to adsorption on foliage and soil (Hensson, 1977; Wall et al., 1981; Bengtsson et al., 1994; Karg et al., 1994). Losses are also due to turbulent winds at the canopy surface, and the antennal signal above the pea canopy showed much stronger fluctuations than within the canopy (Bengtsson et al., 1994).

Caro et al. (1980) measured the vertical distribution of (Z)-9-tetradecen-1-ol formate by air sampling in a corn field. Little vertical mixing of disruptant within the canopy coincided with lower wind speeds, whereas rapid dilution occurred at the canopy top due to higher wind shear. The field EAG apparatus allows sensitive detection of ambient pheromone at a high temporal resolution,

but only a simultaneous input of wind vector data will allow detailed investigations of the dispersal of disruptant chemicals (Koch et al., 1992).

The impact of foliage on the distribution of disruptant chemicals and the success of mating disruption is exemplified by trials with the European grape berry moth, *Eupoecilia ambiguella*, in which reliable control was achieved during the second flight period when the vines were in leaf (Vogt et al., 1993). The presence of vine foliage results in a more homogenous distribution and higher concentrations of pheromone (Karg et al., 1990; Karg and Sauer, 1993). Compared to vineyards or orchards the canopy of pea fields is much denser. This favors maintenance of aerial disruptant concentrations and facilitates the simulation of field treatments in small experimental plots within untreated pea fields (Figure 1).

Aerial Concentrations of Disruptant Chemicals. An essential requirement for pheromone concentration measurements based on EAG recordings is the low response of *C. nigricana* male antennae to nonpheromonal volatiles in pea fields (Bengtsson et al., 1994). By comparison, *Cydia pomonella* and *Epiphyas postvittana* strongly respond to the background odor in orchards (Suckling et al., 1994; Rumbo et al., 1995).

A correlation of *E*8,*E*10–12:Ac stimulus concentrations and EAG amplitudes was used to calculate aerial concentrations from the field EAG recordings (Bengtsson et al., 1994). The average concentration of *E*8,*E*10–12:Ac at 21–36 days after dispenser placement was 1.6 ± 1.2 ng/m^3 (N = 36) in the laminate dispenser treatment and 2.7 ± 1.9 ng/m^3 (N = 36) in the rope dispenser treatment (Table 1). The male antenna also responds to the nonpheromonal isomers *E*,*Z* (55%), *Z*,*E* (27%), and *Z*,*Z* (35%) relative to *E*,*E*, respectively (Bengtsson et al., 1994).

The concentration of *E*8,*E*10–12:Ac in the rope dispenser treatment, obtained by sampling ca. 4 m^3 of field air on Tenax filters, was 1.3 ± 0.5 ng *E*8,*E*10–12:Ac/m^3 (N = 3). This compares well to the results from EAG recordings (Table 1). Aerial *E*8,*E*10–12:Ac concentrations among the laminate dispensers were below the quantitative limit of approx. 1 ng/m^3 (N = 4). Samples of 2 m^3 air from the rope dispenser treatment did not contain detectable amounts of *E*8,*E*10–12:Ac (N = 2). Identity of *E*8,*E*10–12:Ac in the air samples was confirmed by GC/EAD.

Air from pea fields did not contain volatile compounds interfering with *E*8,*E*10–12:Ac on the DB-1 and the DB-Wax columns. Air samples from forests or orchards may contain more nonpheromonal volatile compounds and in larger amounts. Methods to identify and quantify pheromones from more polluted air samples, including clean-up and derivatization procedures, have been developed by Caro et al. (1978, 1979) and Wiesner et al. (1980). For chemical quantification, ambient pheromone must be collected from several cubic meters of air during several hours. Data on time-averaged concentrations are available only

after sample work-up in the laboratory. In contrast, the field EAG apparatus directly visualizes the antennal response to ambient pheromone.

Behavioral Observations

Observations in Untreated Fields. During late afternoons, with the first noticeable decrease in light intensity, males were observed to fly above the pea canopy, frequently changing direction. Visual and olfactory cues from the host plant may contribute to the guidance of these flights, which were apparently not oriented upwind. Such flight activity in the vicinity of food plants is commonly observed in tortricid species (Bradley et al., 1979) and is clearly different from the much slower, anemotactic flight in the proximity of pheromone sources.

Males were attracted to synthetic pheromone during ca. 1 hr prior to the onset of female calling. Cages with females were unattractive at this time (Figure 2). A few minutes before the first female in the cage assumed the typical calling position, the first males were already landing and wing-fanning on the cages. Visibly calling females were more attractive than traps with propheromone sources (Figure 2). Cages with calling females, kept in an air-tight container outside the field, attracted the first male within 1.1 ± 0.4 min ($N = 3$) after they had been placed in the pea field.

We conclude from our observations in the control field that most males are already on their wings when they perceive a calling female. *C. nigricana* females are likely to be mated as soon as they start to release pheromone, and mating success should be highest for males that quickly arrive at the female from a short distance. Male upwind orientation flights over 50 m or more, although readily induced by sources of synthetic pheromone (Wall and Perry, 1980, 1987), would only occur at low population densities.

Observations in Pheromone-Treated Field. During the day after dispenser placement, male moths were attracted to rope dispensers and frequently landed and wing-fanned on surrounding pea plants. Males were observed to spend several minutes searching within one 100-m^2 plot and were also attracted to traps with synthetic pheromone, but only from distances of < 1 m (see also Bengtsson et al., 1994).

After formation of 6% of the antagonistic isomers *E,Z*-, *Z,E*-, and *Z*8,*Z*10-12:Ac, two days after dispenser placement (see above), male attraction to dispensers or prolonged searching within the canopy no longer occurred. Instead, orientation flights were directed towards the traps, sometimes over several meters. Males leaving the traps often flew rapidly downwind and out of the pea field, at least 1 m above the canopy. Males arriving from the outside were repelled at the field border.

Compared to the control field, only few males were observed and attraction to traps was significantly reduced (Figure 2). According to larval counts and

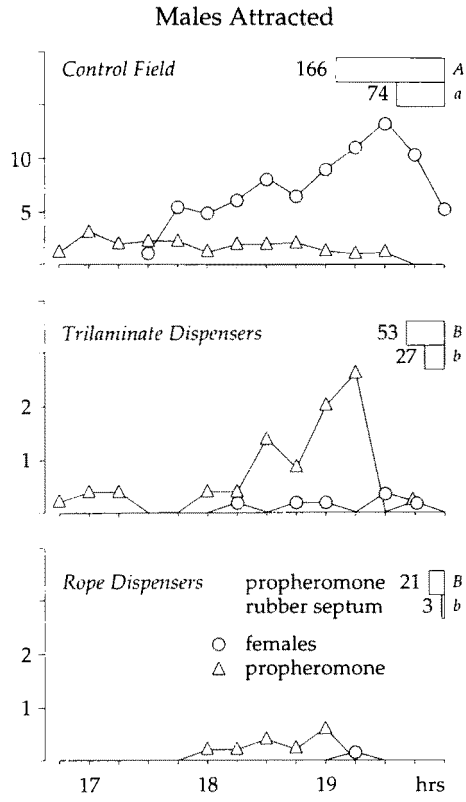

FIG. 2. Observations of *C. nigricana* male attraction to calling females and propheromone sources (circles, triangles) and trap catch (bars) with propheromone sources and *E*8,*E*10–12:Ac on a rubber septum. Average number of males per 15 min per day attracted, in the untreated control field at Höör (three flight periods) and in the trilaminate and rope dispenser treatments in the test field at Veberöd (five flight periods, each), 9–37 days after dispenser placement. The number of males observed in the control field was significantly different from both treatments (Wilcoxon test; $P = 0.05$). Total trap catch of *C. nigricana* males (bars) with 100 μg *E*8,*E*10–12:Ac on a rubber septum or 3 mg propheromone on filter paper, 0–65 days after dispenser placement ($N = 3$; Tukey test; $P = 0.05$).

trapping experiments during the past two years, population densities were similar at Höör (control) and Veberöd (treatment) (Table 1).

In a previous test (Bengtsson et al., 1994), males were not observed at all within the treated field after isomerization of *E*8,*E*10–12:Ac. This was probably

due to higher pheromone concentrations (see below), but the field was also surrounded by dense bushes. At the present study site at Veberöd, males may have traveled downwind 200 m over open farmland from the site of emergence (previous year's pea field) into the treated field.

Male Attraction to Calling Females and Synthetic Pheromone in Relation to Aerial Disruptant Concentrations. Male attraction in the two treatments correlated with pheromone release rate from the attractant source and the background level of disruptant (Table 1, Figure 2). The propheromone traps attracted more males than traps baited with rubber septa or cages with females. Fewer males responded in the rope dispenser than to the trilaminate dispenser section.

At 330 dispensers/ha, the overall release rate was 45 and 14 mg *E*8,*E*10–12 : Ac/ha/day in the rope dispenser and trilaminate dispenser treatments, respectively, 10–34 days after dispenser placement. Aerial concentrations according to EAG measurements were 3 and 2 ng/m^3 (Table 1). During the previous trial (Bengtsson et al., 1994), in which 600 rope dispensers/ha were used (releasing 82 mg *E*8,*E*10–12 : Ac/ha/day, 20 days after dispenser placement), aerial pheromone concentrations ranged from 2 to 7 ng/m^3, and trap catch was completely shut down with 100 μg *E*8,*E*10–12 : Ac on rubber septa.

In the disruption field, calling females were less attractive than propheromone sources. During five calling periods a total of one and six males was attracted to cages with calling females in the rope and laminate dispenser treatments (Figure 2). At aerial concentrations of a few nanograms of Δ8,Δ10–12 : Ac (Table 1), the males remaining in the test field apparently no longer perceived the female signal. Concentrations of ≥ 1.4 ng/m^3 of gossyplure in cotton fields prevented trap catches of *Pectinophora gossypiella* (Flint et al., 1990).

The elevated release rate from propheromone sources (Table 1) may explain the superior attractancy of calling females in the control field (Figure 2). However, a synergistic effect of minor gland components cannot be excluded. These did not increase trap catch when added to *E*8,*E*10–12 : Ac in the female-produced ratios, but an antagonistic effect of *E*9–12 : Ac at 100% addition hints at a behavioral role of this compound (Witzgall et al., 1993).

Female Calling and Mating in Field Cages. Sexual communication over a few centimeters' distance was not disrupted; the mean frequency of matings in cages with females and males was 31% per day in the control (N = 50 females; over three days), and, in the two treatments, 25% (N = 60) and 27% (N = 60). Female calling behavior was unchanged. The average duration of female calling was 51 $\pm$ 29 min (N = 50) in the control field, 61 $\pm$ 27 min (N = 60) among the laminate dispensers, and 58 $\pm$ 36 min (N = 60) among the rope dispensers. Peak calling occurred at 18.45 hr in the control and at 19.00 hr in both treatments. However, it remains to be determined whether ambient pher-

omone modifies premating dispersal of females. Shortly before onset of calling, a high locomotory activity was observed among the caged females, both in the treated and untreated field.

Larval Counts

The test field at Veberöd was isolated from other pea fields. Larval attack was significantly reduced (1.8 ± 0.8%; 6.0 ± 3.2 larvae/m^2) compared to a field at the same site in 1992 (34.1 ± 8.4%; 52 ± 12.8 larvae/m^2) and the control field at Höör (36.2 ± 16.3%; 48 ± 13.0 larvae/m^2). A high density of parasitoids may enhance the efficacy of species-specific control by mating disruption in the following season.

CONCLUSION

The attraction of male pea moths, *C. nigricana*, to calling conspecific females was almost completely eliminated by air permeation with *E*8,*E*10–12 : Ac and its geometric isomers. An off-blend of sex pheromone and attraction antagonists is thus effective in suppressing sexual communication in *C. nigricana*. The significant reduction of larval infestation suggests that the pea moth can be controlled by mating disruption without the use of insecticides.

According to the field EAG recordings, aerial concentrations of at least 2 ng/m^3 of Δ8,Δ10–12 : Ac are required to ensure disruption of mating. At a concentration of 1.6 ± 1.2 ng/m^3 among the trilaminate dispensers, a significant number of males were attracted to traps baited with 100 μg *E*8,*E*10–12 : Ac, and a few males even responded to cages with calling females. In a previous study, trap catch was entirely suppressed with concentrations ranging from 2 to 7 ng/m^3 (Bengtsson et al., 1994). These aerial concentrations were computed from field EAG recordings and can only be considered as estimates, even though their validity is supported by sampling of field air followed by chemical analysis. Furthermore, the EAG data correspond with behavioral observations and trap catch data at different dispenser densities and release rates.

Immediately after placement of the fresh dispensers that were emitting rather pure *E*8,*E*10–12 : Ac, males frequently landed and wingfanned on dispensers and surrounding pea plants. Plumes emanating from traps may have been camouflaged by ambient *E*8,*E*10–12 : Ac, as males were attracted only from a short distance. Thereafter, isomerization of *E*8,*E*10–12 : Ac to *E*,*Z*-, *Z*,*E*-, and *Z*8,*Z*10–12 : Ac had a strong antagonistic effect on male searching within the field. In contrast to a previous trial (Bengtsson et al., 1994), some males remained in the field and were attracted to traps, probably because of lower disruptant concentrations. These males oriented over at least several meters' distance to

sources of *E*8,*E*10–12 : Ac, against a background blend of Δ8,Δ10–12 : Ac. The higher proportional release of the antagonistic isomers by trilaminate dispensers compared to the rope dispensers may have accounted for a satisfactory reduction of larval attack, despite lower release rates.

The time lag between the onset of male responsiveness to pheromone and the onset of female calling may be decisive for mating disruption of *C. nigricana* by Δ8,Δ10–12 : Ac. Males were repelled from the treated field as early as at the beginning of their diel flight period, up to 1 hr before females started to release pheromone. Manipulation of male behaviors during this premating flight activity should be most efficacious when using repellents, rather than attractants. This is particularly important in nonpermanent crops. Peas are sown in different fields every year, precluding moth emergence within the crop. As shown by matings in cages with males and females, it is impossible to impair matings at nanogram aerial concentrations of disruptant if the males are close enough to calling females.

Mating disruption is effective in the grape berry moth *Eupoecilia ambiguella*. Dispenser formulations contain the main pheromone component, *Z*9–12 : Ac, and a few percent of the antagonistic *E* isomer (Arn et al., 1986; Vogt et al., 1993). Mating disruption of the other European grape berry moth, *Lobesia botrana*, by its main pheromone component *E*7,*Z*9–12 : Ac (Arn et al., 1988) is less reliable. This may be due to male attraction to vineyards treated with this compound (see Arn and Louis, 1996), rather than the use of an incomplete pheromone blend.

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A METHOD FOR COLLECTION, LONG-TERM STORAGE, AND BIOASSAY OF LABILE VOLATILE CHEMOSIGNALS

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Abstract—A procedure for headspace sampling and long-term storage of organic volatiles coupled with gas chromatographic-mass spectrometric (GC-MS) analysis was used to study the volatile chemosignals in a biological secretion prior to bioassay. The approach involved collecting the volatiles in evacuated canisters from an apparatus in which 1 ml of secretion was dispersed for headspace sampling. These canisters, stainless steel, 850 ml, and 100% internally electropolished, have been demonstrated to store volatile compounds, in chemically stable form, for several weeks. The GC-MS analyses provided the quantitation and identification of compounds from C_3 through C_{14} at concentrations as low as 0.10 parts per billion volume. The approach was used to study chemosignals of musth temporal gland secretions (TGS) from a male Asian elephant (*Elephas maximus*). Fresh TGS material loses its biological activity within 1 hr. TGS material stored at −20°C usually looses its activity within 30 days. The usefulness of this method for long-term storage of the volatile chemosignals was demonstrated by the retention of biologically active TGS headspace compounds, as determined through bioassays, stored in these canisters for one year.

Key Words—Chemosignal, pheromone, Asian elephant, *Elephas maximus*, temporal gland secretion, musth, avoiding reactions, headspace analysis, 2-hexanone, 3-pentanone, 1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane.

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INTRODUCTION

Temporal gland secretion (TGS) during the condition of musth in the male Asian elephant has been described by Eisenberg et al. (1971), Jainudeen et al. (1972), and Rasmussen et al. (1984, 1990). Chemosignals in TGS produce avoidance reactions in conspecific females (Molamure, 1969; Perrin, 1995; Rasmussen, personal communication). The avoidance reaction by female Asian elephants to fresh TGS is an instantaneous and rapid backing away, involving both trunk and body movements (Perrin, 1995). Our previous evidence indicates that only freshly collected TGS elicits the avoidance reaction in the females. Frozen (−20°C) TGS material and fresh, nonrefrigerated TGS more than 1 hr old does not elicit this response. Olfaction is the primary chemosensory system utilized by the females to detect these compounds (Perrin, 1995). Thus, we hypothesized that the TGS avoidance compounds were volatile and ephemeral. To study these volatile chemosignals, we developed an in situ study approach to collect and store compounds in TGS headspace samples from fresh material for subsequent analyses and bioassay. This study is the first to investigate biologically active compounds from temporal gland secretions of elephants that appear to elicit deterrence.

METHODS AND MATERIALS

Collection of Temporal Gland Secretion. The secretion to be studied, temporal gland secretion (TGS), was obtained in the following manner. Twenty-one samples of 5–7 ml volume of fresh TGS were collected from a 28-year-old male Asian elephant during an entire musth cycle (three months) at the Metro Washington Park Zoo, Portland, Oregon. For collection, the animal was loaded into a squeeze chute (a hydraulic operated restrainer) in the mid-morning and several TGS samples were collected per week throughout the three-month study period. A beaker was placed under the temporal gland (mid-cheek region), and the gland was gently pressed several times causing secretion to flow into the beaker. Immediately after collection, 3 ml of the freshly collected TGS was set aside for bioassay, and 1 ml was placed in the sample collection apparatus for a headspace sample.

Sample Collection Apparatus and Headspace Sampling Procedure. The TGS headspace sampling apparatus (Figure 1) contained two 850-ml stainless steel (Type 304) canisters and a 400-ml glass jar with an air-tight lid perforated with two Swagelok bulkhead O-ring fittings for a gas tight seal. The canisters were 100% internally electropolished by the Summa process (Winberry et al., 1990a) and were fitted with ultraclean Nupro SS-4H4 bellow-stem valves. Electropolishing renders the internal stainless steel surface less reactive by forming an enriched chrome oxide layer so that compounds do not readily adhere or

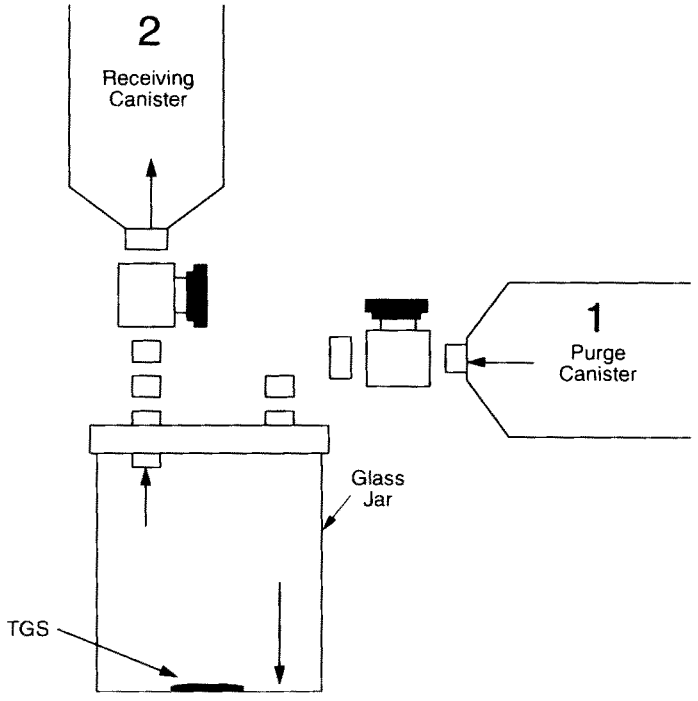

FIG. 1. Diagram of sample collection apparatus showing the positions of the purge canister (canister 1) and the receiving (sample) canister (canister 2). The arrows indicate the directions of airflow.

react to this surface. This improves the recovery of compounds for subsequent chromatographic analyses and bioassay.

The two canisters of the sampling apparatus were the purge canister and the receiving or sampling canister. The purge canister was filled to 30 psig with "zero" air (manufactured air of very high purity) and was connected to the jar at one of the Swagelok fittings, the flushing port, via a 12-in. × 0.25-in. OD piece of Teflon tubing. From this fitting, a glass tube, 0.25-in. OD, extended to within an inch of the bottom of the jar. Although compounds from the ambient air will inevitably show up in the chromatographic analyses due to the sensitivity of the analytical procedure, purging the system with zero air reduces the concentration of these background compounds to insignificant levels. The receiving canister was evacuated to 30 in. Hg vacuum for sample collection and storage and was connected to the other fitting on the jar. This fitting, which is the headspace sampling port, was flush with the inside of the lid, i.e., it did not

extend into the jar, so that the headspace sample was drawn from the top of the jar.

The headspace sampling procedure is described as follows. With the lid removed, 1 ml of fresh TGS, taken directly from the elephant, was placed in the jar. The lid was immediately replaced and fastened. The purge canister was securely fastened to the flushing port, and the receiving canister was connected loosely (finger tight) to the headspace sampling port. The valve of the purge canister was opened, allowing zero air to flow down the glass tube to the bottom of the jar, over the TGS material, and returning to exit at the headspace port. This resulted in a flush to remove any residual air contaminants. The valve of the purge canister was then closed, the connection between the receiving canister and the headspace port was securely tightened, and the TGS compounds were allowed to equilibrate with the headspace. At 10 min, a TGS headspace sample was drawn into the evacuated canister when the valve of this canister was opened.

Upon removal from the jar, the receiving canisters were pressurized with helium to 30 psig to facilitate chromatographic analyses and improve long-term storage. All canister samples were analyzed by a gas chromatograph (GC) that used a flame ionization detector (FID) within one month of collection and then stored for one year at room temperature. The jar, lid, and fittings of the apparatus were washed, rinsed with deionized water, and baked in a high temperature over before reuse. The Teflon tubing of the apparatus was purged with zero air for 5 min, at an elevated temperature (100°C), before reuse.

GC-FID Analysis of Polar Compounds in Dry and Moist Canisters. Although electropolished, the internal walls of the stainless steel canisters still have some degree of adsorption. Compounds, especially polar compounds, will bind to this surface, leading to poor recovery of the sample compounds from the canisters during the chromatographic procedures and bioassays. A moist internal environment greatly improves the recovery of the compounds withdrawn from the canisters. The hypothesized mechanism is that moisture will preferentially bind to the reactive sites on the stainless steel surface, permitting the polar compounds to be much less adsorbed on the internal walls. TGS fluid has a high water content. The water in the TGS very quickly saturated the headspace in the sample jar of the apparatus and would ultimately adhere to the internal stainless steel walls of the sample canisters. Thus, optimum recovery of the TGS headspace compounds during chromatographic analyses was expected. However, we did perform an experiment to demonstrate the necessity of a moist internal canister environment for recovery of polar compounds from a calibration standard prepared in dry nitrogen. This would also be important for the preparation of synthetic mixtures for bioassay.

The procedure for this experiment was as follows. Six canisters, initially under high vacuum, were utilized. Three canisters were injected with 0.5 ml of deionized water and the other three were not. All six cans were subsequently

filled with a polar compound standard in the low parts per billion volume (ppbv) range containing acetone, 2-propanol, 2-butanone, and 1-butanol (this was a National Institute of Standards and Technology standard gas mixture). Each canister was pressurized with helium to 60 psig and the headspace volatiles were analyzed by GC-FID within a 24-hr period. A mean and standard deviation of the peak area response for each compound was determined from multiple analyses.

GC-FID and GC-MS Systems. The gas chromatograph used for this study was a Hewlett Packard 5790A with a flame ionization detector. The column used was a DB-1, 0.32 mm ID × 60 m × 1.0-μm film thickness, polymethyl silicone-coated capillary column (J&W Scientific, Inc.). The FID output was processed via a Hewlett Packard 3392A integrator. The sample introduction system employed a Carle six-port valve in line with a U-tube cryogenic trap (0.125-in. OD × 9-in.) containing 60/80 mesh glass beads. The GC oven was temperature programmed from −60°C to 200°C at 4°C/min, with a 5-min initial hold at −60°C.

More specific analyses were obtained with a gas chromatographic–mass spectrometric (GC-MS) system. This system consisted of a Hewlett Packard 5890A GC and a 5970B Hewlett Packard mass spectrometer. The GC used a DB-1, 0.25 mm ID × 60 m × 1.0-μm film thickness, polymethyl silicone-coated capillary column (J&W Scientific, Inc.). The temperature program and sample introduction procedure was identical to that used in the GC system above. The mass spectrometer was programmed for a mass ion scan of 33–300. This allowed for identification of compounds from C_3 through C_{14} down to levels as low as 0.10 ppbv concentration. Compounds were identified using a NBS 75 K Hewlett Packard MS Chem Station library search and were manually rechecked with NIST/EPA/NIH Mass Spectral Data Base Version 4.01.

Bioassay of Fresh Temporal Gland Secretion. The bioassay of fresh TGS consisted of controlled airflow (zero air) passing over the TGS exiting through a 4-in. opening in the wall of a 20 × 40-ft. room containing four female Asian elephants. The observer had a safe and inconspicuous position with an unobstructed view of all animals via an elevated platform. When an elephant placed her trunk on or near the hole in the wall, airflow over the TGS material was initiated and avoidance reactions were monitored for that animal. A sample was only considered biologically active if at least one animal exhibited instantaneous retreat subsequent to exposure to the air flowing over the fresh TGS. The study time (20 min) was considered complete when all animals were exposed once to the TGS sample. A total of 21 bioassays with fresh TGS were conducted.

GC-MS Analyses of Temporal Gland Secretion Headspace Canister Samples. At the end of a one-year storage period, the behaviorally characterized TGS headspace canister samples (21) were divided into two groups. The bioactive samples (10) were in one group, and the inactive samples (11) were in the other group. The total nonmethane organic carbon (TNMOC) in these samples

was determined using the US Environmental Protection Agency (EPA) Compendium TO12 method (Winberry et al., 1990b). This was done to determine the total concentration of the analytes so that the optimum amount of sample could be loaded on the column for GC-MS analyses. The results of the EPA TO12 analyses indicated that there was not enough material in the canisters for both GC-MS analyses and subsequent bioassays. To increase the amount of material for GC-MS analyses and bioassays, the bioactive canister samples were cryogenically transferred (pooled) into a single 850-ml canister, and the inactive canister samples were pooled into another 850-ml canister. Another TNMOC analysis was conducted on both of these pooled samples, and it determined that there was an adequate quantity of material for both GC-MS analyses and subsequent bioassays. Four micrograms of the pooled active and the pooled inactive TGS headspace samples were each separately analyzed by GC-MS.

Bioassay of Pooled Temporal Gland Secretion Headspace Material. The pooled active and inactive TGS headspace material was bioassayed. Because of the limited amount of material, the original bioassay procedure was modified. We connected a purge-tee assembly, which included a 0–60 psig gauge and a toggle valve, to the canisters and manually placed the canisters under the trunk orifices of the same four female elephants utilized in the initial bioassays. A brief purge of the sample was administered, approximately 400 ml, to the opening of their trunks by depressing the toggle valve lever. A canister of zero air was the control. One bioassay per animal was performed due to the limited availability of sample. These bioassays had a total duration of 5 min.

Preparation of Synthetic Compounds for Bioassay. Ten compounds (purity = 99.8% or higher) that were identified in TGS were bioassayed among three female Asian elephants at the Metro Washington Park Zoo, Portland, Oregon, in 16 bioassay tests. The known compounds were prepared by injecting them into the 850-ml canisters. These canisters were preinjected with 0.50 ml deionized water to ensure that the compounds would not adsorb onto the walls of the canisters. The canisters were then filled with zero air to 30 psig. The compounds were bioassayed at concentrations of approximately 1 parts per million volume (ppmv). Purge-tees were connected to the canisters, and the contents of the canisters were vented to the end of the elephant's trunk. Approximately 400 ml of sample was released per test and responses for each animal were monitored.

Control Samples. Control blanks were run on the headspace of the TGS apparatus. The results demonstrated that contamination from this possible source was minimal. One contaminant, dichloromethane, did show up in these control blanks. Dichloromethane is routinely utilized for organic extractions in our laboratory. The TGS apparatus is stored in our laboratory where high levels of dichloromethane in the lab air probably got into and adhered to the apparatus.

RESULTS

Bioassay of Fresh Temporal Gland Secretion. The bioassays of the 21 fresh TGS samples established that 11 were biologically active, i.e., elicited avoidance reactions, and 10 were inactive (Table 1). During these bioassays, there was individual variation in response to the TGS samples among the four females. Among the 11 samples categorized as bioactive, two females (elephants B and C) responded to nearly all of these samples, one female (elephant A) responded to one sample, and one animal (elephant D) did not respond to any of the samples.

Bioassay of Pooled and Stored Temporal Gland Secretion. The results of the bioassay tests with the pooled TGS headspace material stored for one year were as follows. In all of the bioassay tests performed, no animals responded to the inactive TGS material. Likewise, the zero air control samples elicited no responses. In contrast, three of the four animals displayed an avoidance reaction to the pooled bioactive TGS headspace material (Table 2). The animal that did not avoid the pooled bioactive TGS sample (elephant A) was generally nonresponsive to freshly collected TGS samples, as mentioned above.

GC-FID Analysis of Polar Compound Standards in Dry and Moist Canisters. Four compounds—acetone, 2-propanol, 2-butanone, and 1-butanol—were tested for recovery from moist and dry canisters. The percent recovery of these

TABLE 1. SUMMARY OF 21 BIOASSAYS OF FRESH TGS WITH FOUR FEMALE ASIAN ELEPHANTS[a]

	Backing up responses to TGS sample										
Elephants	1	2	3	4	5	6	7	8	9	10	11
TGS samples scored as biologically active											
A	–		–	–	–	–	–	–	+		
B	+	+	+	+	+	+	+	+		+	+
C	+	–	+	+	+	+	+	+		–	+
D	–		–	–	–	–	–	–	–		
TGS samples scored as biologically inactive											
A	–	–		–	–	–	–	–		–	
B	–	–	–		–	–		–	–		
C	–	–	–		–	–		–	–		
D	–	–		–	–	–	–	–		–	

[a]A, 42; B, 29; C, 30; and D, 9 year-old female Asian elephants; a "+" = avoidance reaction observed, "–" = no avoidance reaction observed, and blanks indicate that the animal was not present.

TABLE 2. SUMMARY OF BIOASSAYS AMONG FOUR FEMALE ASIAN ELEPHANTS WITH POOLED TGS HEADSPACE CANISTER SAMPLES STORED FOR ONE YEAR[a]

Elephants	Backing up responses		
	Bioactive TGS	Inactive TGS	Zero air
A	−	−	−
B	+	−	−
C	+	−	−
D	+	−	−

[a]A, 43; B, 30; C, 31; and D, 10-year-old female Asian elephants; a "+" = avoidance reaction observed, "−" = no avoidance reaction observed. Elephant D was exposed to a male elephant for the first time prior to this experiment.

compounds in the dry canisters was considerably reduced (27% to 93%) in comparison to the percent recovery demonstrated in the moist canisters (Table 3). In addition, in the moist canisters, the standard deviations of the mean recovery of compounds were smaller relative to the standard deviations of the mean recovery of compounds in dry canisters (Table 3). The smaller standard deviations indicate that moisture inside the canisters increases the reproducibility of recovery of compounds during repeated GC-FID analyses.

GC-MS Analysis of Pooled and Stored Temporal Gland Secretion. The results of the GC-MS analyses of the pooled headspace material stored for one year were as follows: The compositions of the pooled TGS samples, as deter-

TABLE 3. COMPOUNDS PRESENT ONLY IN BIOACTIVE TGS HEADSPACE SAMPLES

Compound	Relative retention time (min)	CAS #[a]
3-Methyl-2-butanone	40.10	563-80-4
2-Pentanone	41.90	107-87-9
3-Pentanone	42.70	96-22-0
4-Methyl-2-pentanone	45.30	108-10-1
3-Methyl-2-pentanone	45.90	565-61-7
2-Methyl-3-pentanol	47.80	565-67-3
Cyclopentanone	47.80	120-92-3
3-Hexanone	48.00	589-38-8
2-Hexanone	48.30	591-78-6
1,5-Dimethyl-6,8-dioxabicyclo[3.2.1]octane (frontalin)	57.60	28401-39-0

[a]CAS = Chemical Abstracts Service.

mined by GC-MS, were similar to the compositions of the single TGS samples measured at the time of collection (one year earlier) by GC-FID. Forty-three compounds, ranging from 44 da to 142 da molecular weight, were identified by GC-MS in the biologically active pooled TGS sample (Perrin, 1995). Using GC-FID standards that were calibrated against NIST/SRM of propane and benzene, it was determined that these compounds ranged in concentrations from 2 ppbv to 6 ppmv. Most of the compounds identified were relatively simple ketones, aldehydes, and alcohols. Most of the compounds had a 95% or higher match with the library. Because of the relatively simple nature of the compounds and the accurate retention times, it was not necessary to confirm their identity using known materials. In addition to the 43 compounds identified in TGS, 24 compounds were identified in the background ambient air and are listed in Table 4. These compounds commonly occur in the atmosphere and were generally in concentrations of less than 1 ppbv.

By comparisons of the GC-MS and GC-FID chromatograms, it was established that 10 of the compounds identified in the pooled bioactive TGS sample were present in all 11 single bioactive samples and were not present in any of the 10 inactive samples. These compounds are listed in Table 5 and are shown in the GC-MS chromatogram in Figure 2. The compounds consisted

TABLE 4. TOTAL NUMBER OF AVOIDANCE REACTIONS TO SYNTHETIC COMPOUNDS OF TGS DURING 16 BIOASSAY TESTS WITH THREE FEMALE ASIAN ELEPHANTS

Compound	Number of times compound bioassayed	Number of backing up responses
Compounds present only in bioactive TGS		
2-Hexanone	18	4
4-Methyl-2-pentanone	3	0
2-Pentanone	9	0
3-Pentanone	9	2
Compounds present only in inactive TGS		
Acetic acid	6	0
Compounds present in both bioactive and inactive TGS		
Acetone	12	0
2-Butanone	30	0
Ethanol	9	0
2-Heptanone	27	0
2-Nonanone	15	0
Control		
Zero air	16	0

TABLE 5. GC-FID ANALYSIS OF POLAR COMPOUND STANDARD IN MOIST AND DRY 850-ml INTERNALLY ELECTROPOLISHED STAINLESS STEEL CANISTERS[a]

Compounds	Concentration (ppbv)	Peak area response				% Lost relative to moist canisters
		Moist canisters		Dry canisters		
		Mean	% SD	Mean	% SD	
Acetone	47	45499	9	15055	24	67
2-Propanol	50	24262	15	17791	60	27
2-Butanone	48	81862	6	5500	33	93
1-Butanol	52	32953	11	6806	49	79

[a]This was a National Institute of Standards and Technology standard gas mixture. Mean peak area response values were determined from analyses of three canisters of the standard in both moist and dry canisters. All samples were prepared and analyzed in <24 h. SD = standard deviation.

of eight ketones, one alcohol, and one terpene (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane, commonly known as frontalin). The identification of frontalin was confirmed by GC-MS with a frontalin standard (Figure 3).

Bioassay of Synthetic Compounds Identified in Temporal Gland Secretion. The 10 synthetic compounds that were bioassayed included four compounds that were present only in active TGS (Table 6). Among these four compounds, two compounds, 2-hexanone and 3-pentanone, elicited avoidance reactions in the females. All of the other compounds, including the zero air controls, did not elicit any avoidance reactions during 16 bioassay tests with three female Asian elephants.

DISCUSSION

This is the first study to demonstrate avoidance by female Asian elephants to conspecific male musth temporal gland secretions in a series of controlled experiments. This is also the first study where a biologically active component of temporal gland secretion was collected and stored in the gas phase. Our approach resulted in the identification of at least two possible avoidance chemosignals in TGS. This was possible in part because of the use of a methodology that allowed the stored volatiles to retain biological activity for bioassay.

The retention of biological activity upon long-term storage in the canisters was verified in that three of four elephants avoided the stored bioactive TGS sample when presented with it. The fact that the sample did not elicit avoidance in one of the females (elephant A) may have been related to her dominant

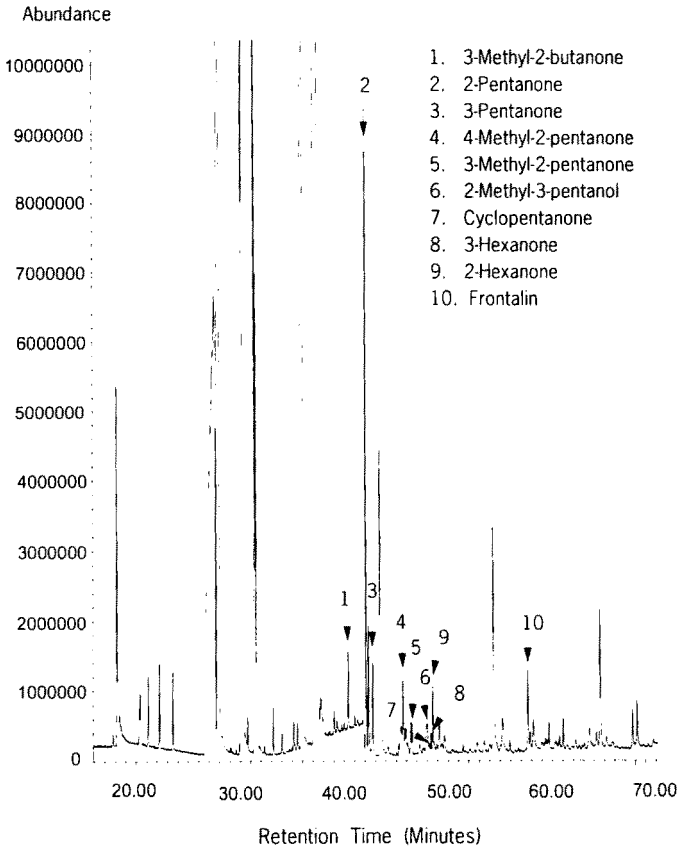

FIG. 2. GC-MS total ion chromatogram of the headspace of biologically active TGS. The 10 compounds listed were present in only the headspace of bioactive TGS samples. These 10 compounds were within an approximate concentration range of 2–130 ppbv.

position. For example, in previous studies, females of subordinate rank responded to a natural component of TGS, cyclohexanone, more frequently than dominant females (Perrin and Rasmussen, 1994; Perrin, 1995).

Additional factors that may affect responses to TGS include estrous status and experience with a bull or sexual maturation (Perrin, 1995). The youngest female (elephant D) did not respond to any fresh TGS samples in the first set of bioassays tests. However, one year later she exhibited avoidance to the stored sample. In the year that elapsed between presentations of the two types of TGS samples, this young female had several exposures to a bull in musth for the first

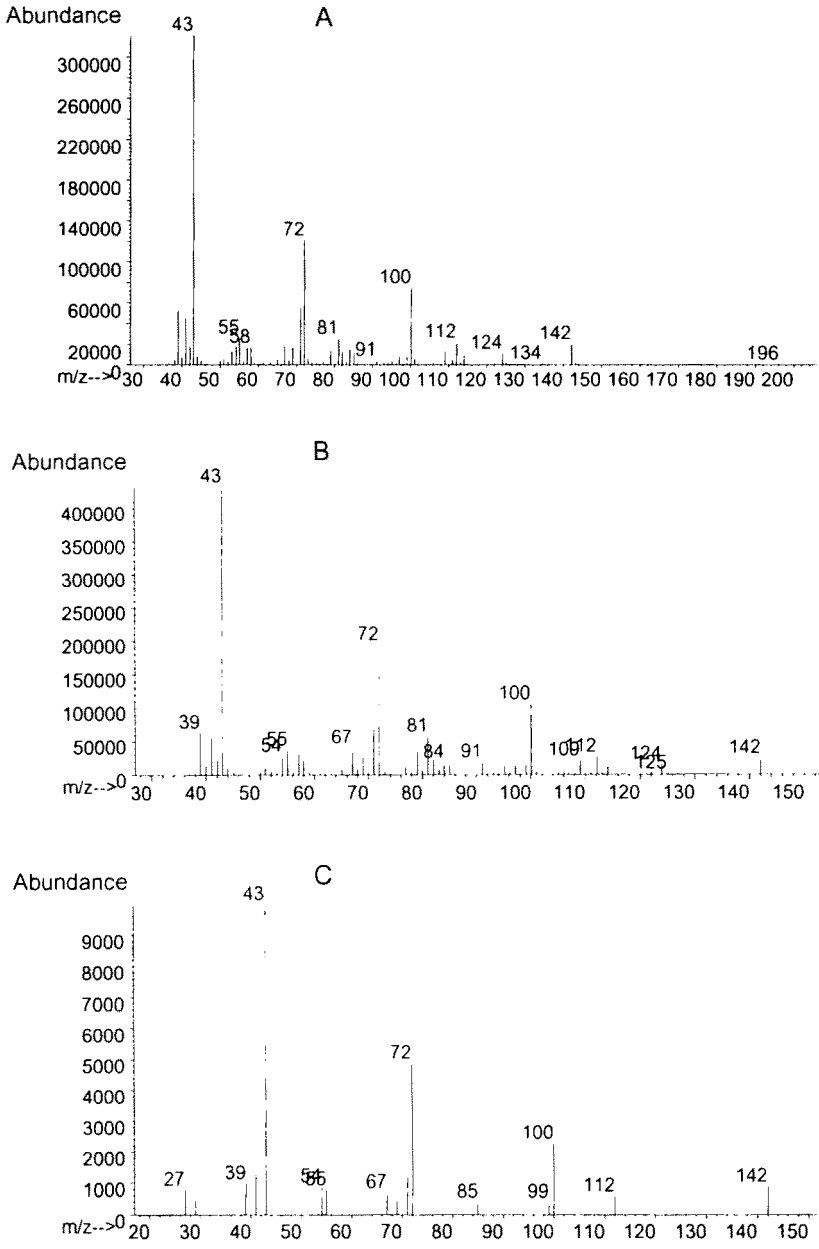

FIG. 3. GC-MS spectra of compound in the headspace of pooled bioactive TGS sample (A), a frontalin standard (B), and the frontalin library match (C) from Hewlett Packard MS ChemStation with a NBS 75K library.

TABLE 6. COMMON ATMOSPHERIC GASES IDENTIFIED IN BACKGROUND AIR OF TGS HEADSPACE SAMPLES[a]

Compound	Common name	CAS #[b]
Carbonylsulfide	OCS	463-58-1
Propene		115-07-1
2-Methyl propane	Isobutane	75-28-5
2-Methyl-1-propene	Isobutene	115-11-7
1-Butene		106-98-9
Butane		106-97-8
trans-2-Butene		624-64-6
cis-2-Butene		590-18-1
Acetonitrile		75-05-8
Trichlorofluromethane	Freon 11 (F-11)	75-69-4
Pentane		109-66-0
2-Methyl-1,3-butadiene	Isoprene	78-79-5
1,1-Dichloroethene	Vinylidenechloride	75-35-4
1,1,2-Trichloro-1,2,2-trifluorethane	Freon 113 (F-113)	76-13-1
Benzene		71-43-2
Heptane		142-82-5
Methylbenzene	Toluene	108-88-3
1,4-Dimethylbenzene	*p*-Xylene	106-42-3
1,2-Dimethylbenzene	*o*-Xylene	95-47-6
Nonane		111-84-2
1,2,4-Trimethylbenzene		95-63-6
Decane		124-18-5
Undecane		1120-21-4

[a]Compounds are listed in order of elution from the GC column and were generally in concentrations of less than 1 ppbv.
[b]CAS = Chemical Abstracts Service.

time. Thus, avoidance to TGS may be a complex learned behavior that is related to dominance, sexual maturation, experience with a male elephant, and perhaps other factors.

The collection and storage of the TGS headspace volatiles allowed us to characterize possible avoidance compounds. We hypothesized that one or more of the 10 compounds present only in the bioactive TGS elicited avoidance. This hypothesis was supported by the results of initial bioassays with single synthetic compounds demonstrating that 2-hexanone and 3-pentanone elicited avoidance. Although 4-methyl-2-pentanone and 2-pentanone did not elicit avoidance reactions when bioassayed as singlets, it is possible that they may augment avoidance elicitation in combination with other compounds.

Two possible bioactive compounds and an unusual terpene, frontalin, were

identified in TGS. It was surprising to find frontalin in male Asian elephant temporal gland secretion because it had not previously been identified in a mammalian secretion. Interestingly, this terpene, a bicyclic ketal, is a well-established aggregation pheromone in bark beetles (Kinzer et al., 1969; Lindgren, 1992). Although frontalin has not yet been bioassayed, it may be involved in avoidance elicitation either as a single compound or in combination with other compounds. In addition, this compound may provide insight into unusual biosynthetic pathways of compounds in TGS.

This study was possible because: (1) a sampling method was developed to collect the headspace volatiles from temporal gland secretion material immediately after it was obtained; (2) the bioassay procedure provided reliable behavioral information allowing discrimination between bioactive and nonbioactive TGS; (3) the internal electropolished surface in the Summa canisters provided sufficient inertness to allow labile compounds in the volatile chemosignal to be stable for up to one year before bioassay; (4) the volume of the canisters allowed for sufficient amounts of headspace sample to be collected for both analyses and bioassay of the TGS material; and (5) the importance of moisture inside the canisters for optimum recovery of compounds for analyses and bioassay also was verified in this experiment.

The points stated above highlight the characteristics and advantages of this new method. Because of the general similarities among most mammalian secretions, e.g., high water content and complex chemical compositions, we suggest that this method would be applicable to the study of chemosignals from a variety of mammalian secretions.

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DAILY RHYTHMS OF METABOLIC RATES: ROLE OF CHEMICAL SIGNALS IN COEXISTENCE OF SPINY MICE OF THE GENUS *Acomys*

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Abstract—The common spiny mouse *Acomys cahirinus* and the golden spiny mouse *A. russatus* coexist in the extreme arid and hot parts of the Arava Rift Valley in Israel. The coexistence of these two species is through exclusion of *A. russatus* from nocturnal activity by *A. cahirinus*, which is nocturnal. An attempt was made to study the daily activity rhythm response of *A. russatus* to chemical signals released by *A. cahirinus*, in order to understand the mechanism of segregation. For this purpose oxygen consumption, $\dot{V}O_2$, was studied in individuals of *A. russatus* kept alone in a metabolic chamber in a constant ambient temperature of 32°C under two photoperiod regimes: 12L:12D, and constant darkness (D:D). After three days of $\dot{V}O_2$ recording under these conditions, chemical signals released from *A. cahirinus* were passed through the metabolic chamber. This treatment caused a shift in the activity rhythm only of *A. russatus* kept at 12L:12D, while a decrease in $\dot{V}O_2$ rates of *A. russatus* was noted in both groups. These results suggest that chemical signals released by *A. cahirinus* may play a role in shifting *A. russatus* toward its diurnal activity.

Key Words—Arid rodents, circadian rhythm, chemical signals, coexistence, metabolic rates, phase shift, oxygen consumption.

INTRODUCTION

The spiny mice of the genus *Acomys* are common murids inhabiting the arid parts of Israel. Two species, the common spiny mouse, *A. cahirinus*, and the

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golden spiny mouse, *A. russatus*, are sympatric and coexist in the same rocky habitat. Although the golden spiny mouse is limited in its distribution to the most extreme hot and dry parts of the Rift Valley and in wadis running into it, the common spiny mouse is distributed in the steppe regions of the Negev highlands and even in the Mediterranean mesic parts of the country (Harrison and Bates, 1991).

In the rocky habitats of the Rift Valley, where both species are sympatric, it was discovered that their coexistence is possible due to competitive exclusion of *A. russatus* from nocturnal activity by *A. cahirinus* (Shkolnik, 1966, 1971). The removal of *A. cahirinus* from the habitat caused a change in the activity pattern of *A. russatus*, which became nocturnal (Shkolnik, 1971). Shkolnik (1966) and Shkolnik and Borut (1969) suggested that certain characteristics of *A. russatus*, such as low metabolic rate, a high lower critical temperature, and a concentrated urine, enable it to cope with an extreme hot and arid environment as a diurnal species.

In recent observations in our laboratory (Rubal et al., 1992) it was determined that when kept alone in a cage under laboratory conditions, *A. russatus* exhibits the activity pattern of a nocturnal rodent. An open question on the relations between these two sympatric species is the mechanisms used by *A. cahirinus* to exclude *A. russatus* from nocturnal activity.

The social response of rodents to chemical signals and its ecological significance has been studied in many species (Stoddart, 1980; Rozenfeld *et al.*, 1987). Phase response curves for social entrainment were reported by Mrosovsky (1988) for the golden hamster *Mesocricetus auratus*. In his opinion social interactions and other disturbances can be mediated through an oscillator phased by general arousal.

This study investigated the phase response of *A. russatus* to chemical signals released by the urine and feces of *A. cahirinus* to assess the mechanism for the competitive exclusion of *A. russatus* from nocturnal activity by *A. cahirinus*.

METHODS AND MATERIALS

Measurements were carried out on six individuals of *A. russatus* kept under a photoperiod regime of 12L:12D and six under free running rhythm (FRR) conditions with a constant dim red light. These individuals were obtained from our breeding colony, in which the ancestors were brought from the Dead Sea area, where both species coexist.

Activity was monitored by the daily rhythms of oxygen consumption, ($\dot{V}O_2$), which was measured by using an open flow system (Depocas and Hart, 1957; Hill, 1972). A flow of 600 ml/min of air dried by silica gel (TamRod)

was used. VO_2 was monitored from air leaving the metabolic chamber by an applied Electrochemistry S-A3 oxygen analyzer. The results were recorded continuously on a Tek-dyn 712 recorder.

The tested individual was kept in a metabolic chamber with a volume of 2.5 liters. The metabolic chamber was kept inside a Forma growth chamber with controlled temperature and photoperiod. The mice were kept at an ambient temperature (T_a) of 32°C, which is the lower critical point of *A. russatus* (Haim and Borut, 1981) and with a photoperiod of 12L : 12D or under constant darkness (FRR).

Rat pellets and carrots were offered at the beginning of each session of measurements and each session lasted for six days. The daily rhythms of $\dot{V}O_2$ were recorded for three days. After this period, a column containing feces and filter paper saturated with urine of *A. cahirinus* was connected to the system just before the flowmeter, and $\dot{V}O_2$ measurements continued for another three days. The urine and feces were added 2–3 hr after the lights went on and were changed daily after 24 hr.

$\dot{V}O_2$ was calculated for every hour of measurement, and all results were corrected to STPD. All $\dot{V}O_2$ patterns were analyzed by the fit cosinor model (Minors and Waterhouse, 1989). A paired *t* test was used in order to establish statistical differences between the results obtained for the mice before and after exposure to the chemical signals.

RESULTS

Reproducible rhythms of $\dot{V}O_2$ were recorded in all measured golden spiny mice. In such mice, the best-fit *tau* analysis revealed a major significant daily rhythm of 24 hr.

Before exposure to chemical signals from *A. cahirinus*, individuals of *A. russatus* kept under a photoperiod regime of 12L : 12D displayed a nocturnal activity pattern. After exposure to chemical signals in urine and feces of *A. cahirinus*, the *A. russatus* mice showed an advance of 3 hr in their activity. Oxygen consumption in mice exposed to the chemical signals showed a tendency to increase as early as 10:30–12:00 hr, while in those not exposed to the chemical signals, the increase began at 13:30–14:00 hr. A significant ($P < 0.001$) advance was noted also in the acrophase (the peak of oxygen consumption). When the *A. russatus* mice were exposed to the chemical signals, the acrophase of oxygen consumption was shifted to 21:40 ± 00:12, while before exposure it was at 00:25 ± 00:11 (Figure 1). Moreover, $\dot{V}O_2$ during maximal activity was about 20% lower in *A. russatus* exposed to chemical signals of *A. cahirinus*.

A. russatus kept under FRR conditions did not respond to the chemical signals of *A. cahirinus* by a phase shift. The addition of the chemical signals

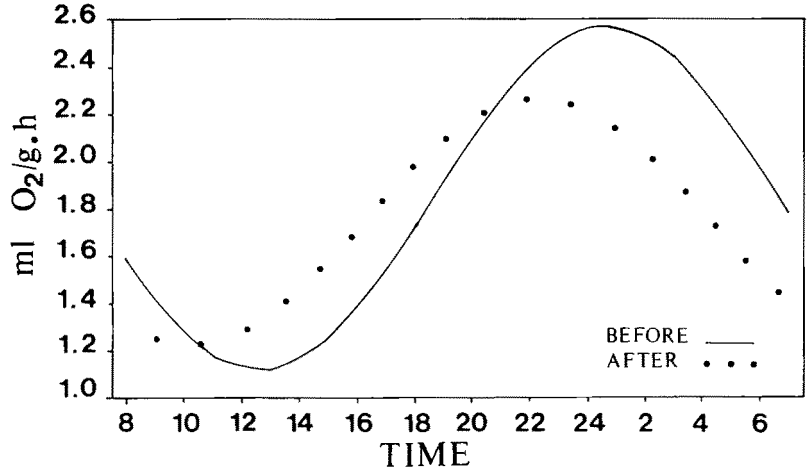

FIG. 1. A cosinor graph describing the average daily rhythm of oxygen consumption ($\dot{V}O_2$) ml O_2/g/hr measured in *Acomys russatus*, kept under a photoperiod regime of 12L : 12D. The solid line describes the rhythm before the addition of the chemical signal released by *A. cahirinus*. The dotted line describes the rhythm after the addition of the chemical signal.

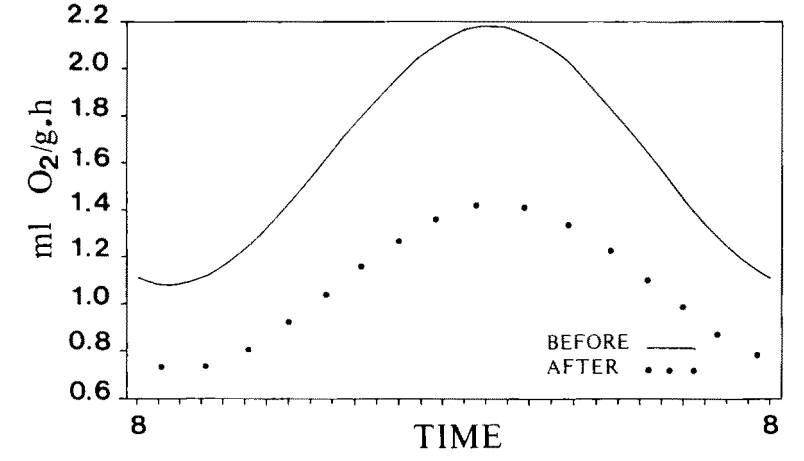

FIG. 2. A cosinor graph describing the average rhythm of oxygen consumption ($\dot{V}O_2$) ml O_2/g/hr measured in *Acomys russatus*, kept under free running conditions. The solid line describes the rhythm before the addition of the chemical signal released by *A. Cahirinus*. The dotted line describes the rhythm after the addition of the chemical signal.

caused a change in their pattern of activity, and several peaks occurred during the activity period, which did not fit the cosinor curve. However, the maximal $\dot{V}O_2$ value (amplitude) of 2.21 $\pm$ 0.2 ml O_2/g/hr was significantly ($P < 0.001$) higher in mice kept alone than in the same mice after exposure to chemical signals of *A. cahirinus*, whereupon the value was only 1.41 $\pm$ 0.3 ml O_2/g/hr (Figure 2). The difference in the maximal values during activity was about 60% and during the resting period only 30%.

DISCUSSION

The desert environment is characterized by low precipitation, which is highly variable and infrequent, with a large unpredictable component. Water is the most important controlling factor for most of the biological processes occurring in such ecosystems (Noy-Meir, 1973). The Arava Rift Valley is a part of the Great Palaearctic deserts, and, apart from its aridity, it is also a very hot desert. To maintain water balance, small mammals inhabiting such arid environments are nocturnal. As such they escape the heat of the day. One of the best-studied examples is the kangaroo rat, *Dipodomys merriami* (Schmidt-Nielsen, 1964).

However, an exception to this behavioral pattern is displayed by the golden spiny mouse, *A. russatus*, a diurnal murid. The diurnal activity of this species results from exclusion by the common spiny mouse *A. cahirinus* (Shkolnik, 1971). The "competitive exclusion principle" formulated by Gause (1934) makes it clear that two species of the same genus cannot occupy identical niches. Furthermore they displace each other, so that one species can take possession of a peculiar mode of life or kind of food, which gives it an advantage over its competitor. The coexistence of these two *Acomys* species is an outstanding example of such relations. Removal of *A. cahirinus* from the common habitat caused a shift in the activity pattern of *A. russatus*, which became nocturnal (Shkolnik, 1971).

Endotherms generally possess a long-term stable mean body temperature with a circadian (24 hr) variation (Aschoff, 1982; Kenagy and Vleck, 1982; Haim and Levi, 1990). The circadian rhythm of body temperature is regulated by a combination of heat production and heat dissipation. In the pouched mouse, *Saccostaomus campestris*, the daily rhythm of $\dot{V}O_2$ is the major metabolic and thermoregulatory rhythm (Haim *et al.*, 1989).

The results of our study reveal that when *A. russatus* is kept alone in the metabolic chamber under a 12L : 12D photoperiod regime or even in free running rhythm (FRR) conditions, it shows a pattern of $\dot{V}O_2$ characteristic of a nocturnal rodent. This result is consistent with those of Shkolnik (1971) from a field study in which *A. cahirinus* was removed from the common habitat and *A. russatus* under such conditions became nocturnal.

Our results also demonstrate that under both photoperiod acclimation conditions, *A. russatus* responded to chemical signals released by *A. cahirinus* by decreasing the $\dot{V}O_2$ values. These results suggest that in the presence of odors released by *A. cahirinus*, *A. russatus* decreases its activity. This behavioral pattern should be further studied.

The addition of chemical signals from *A. cahirinus* caused a shift in the activity of *A. russatus* kept under a 12L:12D photoperiod regime. By contrast, individuals kept under FRR conditions did not respond by a shift of activity. These results suggest that the endogenous oscillator of *A. russatus* is entrained by photoperiod, and under such conditions it may also entrain its daily rhythm of activity by chemical signals released by *A. cahirinus*. In the absence of photoperiodic cues, the oscillator will not respond to the chemical signals released from *A. cahirinus* with a shift in activity.

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NEUTRAL LIPIDS IN SNAIL-CONDITIONED WATER FROM *Biomphalaria glabrata* (GASTROPODA: PLANORBIDAE)

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Abstract—Thin-layer chromatography was used to study neutral lipids in snail-conditioned water (SCW) from *Biomphalaria glabrata* snails. A major lipid fraction in SCW at 2 and 4 hr after snail incubation contained free fatty acids. Fatty acids released per snail per milliliter of water when 10 snails were incubated in 5 ml of deionized water for 4 or 2 hr was 2.4 ± 0.4 μg/ml and 0.9 ± 0.1 μg/ml (mean ± SE), respectively. The amount released at 4 hr was significantly greater than at 2 hr. Snails also released free sterols, a sterol ester–hydrocarbon fraction, methyl esters, and other unidentified lipid fractions into the water. The potential of these neutral lipids to serve as chemoattractants for larval trematodes or snails remains to be determined.

Key Words—Snail-conditioned water (SCW), neutral lipids, free fatty acids, *Biomphalaria glabrata*, TLC, densitometry.

INTRODUCTION

The medically important planorbid snail, *Biomphalaria glabrata*, releases substances into water that attract larval parasites or conspecific snails (Chernin, 1970; Marcopoulos and Fried, 1994). Water containing these substances has been referred to by numerous workers as snail-conditioned water (SCW). SCW contains numerous substances released by the snail from the mouth, anus, nephridiopore, genital openings, body surface, and other structures including nitrogenous wastes, fecal matter, gametes, mucus, and other substances. To

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date, most studies have examined SCW in its entirety, mainly to determine its role as a chemoattractant for larval trematodes or for cohort species of snails.

Except for a recent study by Haas et al. (1995) on SCW from *Lymnaea stagnalis* snails, qualitative and quantitative analyses of the chemicals in SCW are not available. The purpose of this study was to determine the composition of neutral lipids released into deionized water by *B. glabrata* and to quantify by densitometric TLC the major free fatty acid fraction.

METHODS AND MATERIALS

Sample Preparation. Stock cultures of *B. glabrata* were maintained in the laboratory on a mixed diet of boiled leaf lettuce and Tetramin tropical fish food (Tetra Werka, Melle, Germany) (Duncan et al., 1987). To obtain SCW, 10 snails with shell diameters of 7–10 mm were placed in 10-ml beakers containing 5.0 ml of Milli-Q purified water (Millipore Co., Bedford, Maine) for either 2 or 4 hr. Aliquots of 1 ml were removed by pipet from the beaker at 2 or 4 hr and placed in vials. This procedure was repeated 12 times to get eight samples at 2 hr and four samples at 4 hr. Lipids were extracted from each sample with 2.0 ml of chloroform–methanol (2 : 1). The upper aqueous fraction was removed with a pipet and discarded. The lower lipid fraction was evaporated to dryness using a stream of nitrogen gas, and the residue was immediately reconstituted with 100 μl of chloroform–methanol (2 : 1).

HPTLC of Lipids. TLC analysis was performed using Whatman LHP-KDF high-performance preadsorbent silica gel plates (Clifton, New Jersey). Each plate was 10 × 20 cm and contained 19 individual lanes. The standard used to analyze the sample lipids was the neutral lipid standard 18-4A, which contains 0.2 μg/μl each of cholesterol, oleic acid, triolein, methyl oleate, and cholesteryl oleate (Matreya, Pleasant Gap, Pennsylvania).

Various aliquots (1, 2, 4, 8, and 16 μl) of the standard and reconstituted SCW extract were spotted on the preadsorbent area of the TLC plate using a 25-μl Drummond digital microdispenser. The plate was dried with a hair dryer and placed in a paper-lined, glass Camag Twin-Trough HPTLC chamber containing the mobile phase petroleum ether–diethyl ether–acetic acid (80 : 20 : 2). The plate was allowed to develop until the solvent front reached 7.5 cm past the preadsorbent-silica gel interface. Plates were air dried, and the lipids were detected as blue spots on a yellow background after spraying with 5% ethanolic phosphomolybdic acid (PMA) and heating for 15 min at 115°C. Qualitative identification of sample zones was based on migration comparisons with the zones in the lipid standards.

The free fatty acids were quantified by scanning with a Shimadzu CS 930 densitometer operated in single-beam, reflectance mode at a wavelength of 700 nm. The oleic acid standard zones were scanned, and calibration curves relating

scan area and weight spotted were generated from a Quattro 5.0 linear regression program. The single free fatty acid sample zone that had a visual intensity closest to that of the middle standard was measured by scanning, and the weight of free fatty acids for that zone was determined by interpolating from the calibration curve. The amount of free fatty acid in the 1-ml aliquot of SCW was calculated from the following equation: μg free fatty acid in 1 ml SCW = $(W \times R)/S$, where W is the interpolated weight of the free fatty acid in the sample zone, R = 100 μl (the sample reconstitution volume), and S is the volume of sample spotted. The amount of free fatty acid in the original 5 ml of SCW was obtained by multiplying the value obtained by 5.

RESULTS

Based on 12 trials, a major lipid component of SCW for both 2- and 4-hr samples was determined by TLC analysis to be free fatty acid (R_f = 0.43). Figures 1 and 2 show chromatograms of 2- and 4-hr SCW extract, respectively. A sterol ester–hydrocarbon fraction (R_f = 0.84) and a methyl ester-like fraction (R_f = 0.73) were also present but were not further characterized. Trace amounts of free sterol (R_f = 0.29) were also apparent, particularly at 4 hr (not clearly shown in the figures). In Figure 2, a small amount of an undetermined band (R_f

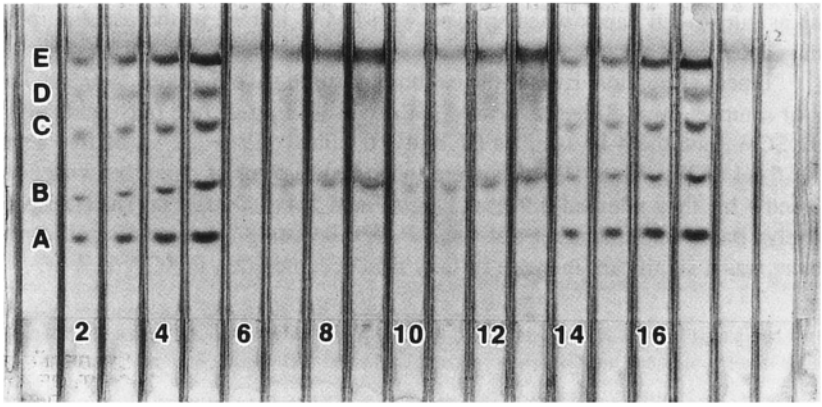

FIG. 1. Photograph of a chromatogram showing 1, 2, 4, and 8 μl of standard 18-4A in lanes 2–5 and in lanes 14–17. Lanes 6–9 contain 2, 4, 8, and 16 μl of extract, respectively, of a sample of water conditioned with *Biomphalaria glabrata* for 2 hr. Lanes 10–13 contain 2, 4, 8, and 16 μl of extract, respectively, of a second sample of water conditioned for 2 hr. Lane 18 contains 8 μl of an extract of deionized water. Abbreviations: A, free sterols; B, free fatty acids; C, triacylglycerols; D, methyl esters; E, sterol esters.

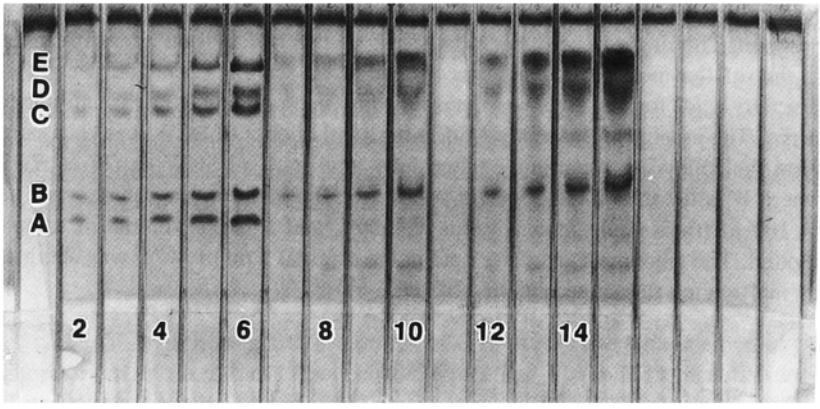

FIG 2. Photograph of a chromatogram showing 1, 2, 4, 8, and 16 μl of standard 18-4A in lanes 2–6. Lanes 7–10 contain 2, 4, 8, and 16 μl of extract, respectively, of a sample of water conditioned with *Biomphalaria glabrata* for 4 hr. Lanes 12–15 contain 2, 4, 8, and 16 μl of extract, respectively, of a second sample of water conditioned for 4 hr. Lane 16 contains 8 μl of an extract of deionized water. Abbreviations: A, free sterols; B, free fatty acids; C, triacylglycerols; D, methyl esters; E, sterol esters.

= 0.59) appeared between the free fatty acid and triacylglycerol (R_f = 0.63) fractions. There were unidentified lipids (R_f = 0.17; R_f = 0.26) that showed up as purple–red bands when sprayed with PMA, instead of the usual purple–blue color.

Linear regression data for the calibration curve gave R values > 0.98. The 2-hr samples (N = 8) had a mean ± SE of 46 ± 3 μg of free fatty acids per 5 ml SCW, and the 4-hr samples (N = 4) contained 119 ± 22 μg of free acids per 5 ml SCW. When 10 snails were incubated in 5 ml of deionized water for 2 and 4 hr, they released 0.9 ± 0.1 μg/ml and 2.4 ± 0.4 μg/ml/snail, respectively. Based on Student's t test (with $P < 0.05$ being considered significant), there was a significant increase in fatty acid accumulation in SCW at 4 hr.

DISCUSSION

Fried and Sherma (1990) reviewed the salient literature on the lipid composition of *B. glabrata* and the medical significance of this snail as a vector of the human blood fluke, *Schistosoma mansoni*.

This study provides quantitative data for the first time on free fatty acids released into water conditioned by *B. glabrata*. Information obtained from this study may be useful in designing a bioassay to test free fatty acids as potential chemoattractants for larval trematodes or snails.

The petroleum ether–diethyl ether–acetic acid (80:20:2) solvent system was suitable for TLC densitometric analyses of the free fatty acid fraction. However, it was not suitable for quantifying other neutral lipids, e.g., the sterol ester–hydrocarbon fraction or the methyl ester-like fraction that appeared as significant components in SCW. Another solvent system, e.g., *n*-hexane–petroleum ether–ethyl ether–acetic acid (50:20:5:1), as described by Smith et al. (1995), may be suitable for further characterization and possible quantitative analysis of these fast-moving zones.

The purple–red zones that appeared following detection of the neutral lipids with PMA could not be identified. In the more than 15 years of experience with PMA as a detection method, two of the authors (B.F. and J.S.) have not seen this reaction on silica gel plates sprayed with PMA.

The accumulation of chemicals in *B. glabrata* SCW will depend on the snail strain, age, diet, and amount of time the snail has spent in the water. In practice, SCW is collected in different ways, over various time periods. For instance, Roberts et al. (1979) collected SCW by maintaining *B. glabrata* snails in distilled water (2 snails/ml) for 5 hr, whereas Marcopoulos and Fried (1994) maintained this snail in chemically defined artificial spring water for 4 hr (1 snail/200 μl). The length of time used to collect SCW was a factor in quantifying neutral lipid components in this work, and about three times the amount of free fatty acids was present at 4 hr compared to 2 hr. Because workers collect SCW at different times (anywhere from 2 to 24 hr after snails are placed in water), the time of sample collection must be considered an important variable when identifying and quantifying components in SCW.

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ANALYSIS OF CHARACTERISTIC HUMAN FEMALE AXILLARY ODORS: QUALITATIVE COMPARISON TO MALES

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Abstract—Odors produced in the human female axillae are of both biological and commercial importance. Several studies have suggested that extracts from female underarm secretions can alter the length and timing of the female menstrual cycle. In addition, more than 1.6 billion dollars are spent annually on products to eliminate or mask the axillary odors. Our recent studies have determined that the characteristic axillary odors in males consist of C_6–C_{11}, saturated, unsaturated and branched acids, with (*E*)-3-methyl-2-hexenoic acid (3M2H) being the major compound in this mixture. The 3M2H appears to be carried to the skin surface bound to two proteins in the axillary secretions. Data reported here show that the same mixture of odorous compounds is found in female axillary secretions, with several minor qualitative differences. Separation of the female apocrine secretions into aqueous and organic soluble fractions demonstrated that 3M2H, and several other members of the acids in the characteristic odor, are released by hydrolysis with base. Electrophoretic separation of the proteins found in the aqueous phase of female apocrine secretions revealed a pattern identical to that seen in males. The qualitative similarity of the acidic constituents making up the characteristic axillary odors of both females and males as well as the proteins present in the aqueous phase suggest a similar origin for axillary odors in both sexes.

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Key Words—Axillary odors, human females, 3-methyl-2-hexenoic acid, androstenol.

INTRODUCTION

Odors produced in the human axillae are of both biological and commercial importance. For example, studies performed in several laboratories have suggested that extracts made from human axillary secretions can alter the length and timing of the human menstrual cycle (Russell et al., 1980; Preti et al., 1986; Cutler et al.; 1986; Stern and McClintock, 1993). In addition, in the United States alone, more than 1.6 billion dollars are spent annually on products to eliminate, mask, or prevent the formation of axillary odors (Ramirez, 1990).

Prior to our studies, investigators examining the nature of axillary odors emphasized the steroid biochemistry occurring in the underarm (Labows, 1988; Gower and Ruparelia, 1993). Radioimmunoassay and gas chromatography–mass spectrometry techniques have been used to identify and trace the origins of volatile, odoriferous steroids in the underarm, such as 5α-androst-16-en-3α-ol (androstenol) and 5α-androst-16-en-3-one (androstenone). The musky, urine-like odors were thought to resemble the odor produced in the axillae (Gower et al., 1994). However, recent studies that employed organoleptic evaluation of gas chromatographic eluants and other analytical techniques, particularly GC-MS, to examine the axillary odor from a combined group of males found that the characteristic odors consist of C_6–C_{11}, saturated, branched, and unsaturated acids, with (*E*)-3-methyl-2-hexenoic acid (*E*-3M2H) being the major component of this mixture (Zeng et al., 1991). The *Z* isomer is also present at about 1/10 the level of the *E* isomer. In terms of relative abundance, these acids, particularly *E*- and *Z*-3M2H are present in far greater quantity than those odoriferous steroids within the acid fraction of the combined male samples; the neutral and basic compounds in these male extracts had little to no odor (Zeng et al., 1991, 1992). Both *E*-3M2H and *Z*-3M2H, as well as a number of other axillary odor components, appear to be carried to the axillary skin surface bound to water-soluble proteins found in apocrine secretions (Zeng et al., 1992; Spielman et al., 1995).

Female axillary secretions and odors have been examined organoleptically and microbiologically (Leyden et al., 1981). These studies suggest that males and females with the same axillary microflora will produce qualitatively similar axillary odors. A greater number of males appear to have the *Corynebacteria* as axillary residents; these bacteria are associated with the strongest axillary odors (Leyden et al., 1981). However, psychophysical studies suggest that the assignment of gender based upon axillary odor is most likely done by intensity and not by overt qualitative differences (Russell, 1976; Doty, 1981).

Studies have examined the volatile steroids present in the female axillary

extracts and made quantitative comparisons in the levels of these to males (Gower et al., 1985; Preti et al., 1987). Our earlier investigation of axillary extracts also suggested that at least one volatile steroid, androstenol, appears to be present in greater concentration during the follicular phase of the menstrual cycle than at any other time (Preti et al., 1987). However, no analyses of secretions from the female axillae have been done to determine whether females contain the same array of acidic compounds in their characteristic odor components. Results of analyses described below demonstrate that both males and females are very similar, although some qualitative distinctions are present.

METHODS AND MATERIALS

Subjects and Collection of Axillary Secretions. Six healthy female donors (ages 24–34; mean = 28.70) were employed to collect the axillary secretions used in this study. The axillae of each subject was sampled to determine its bacterial microflora prior to secretion collection (Leyden et al., 1981). Each subject was found to have lipophilic diphtheroids as normal axillary residents. The latter have been associated with the production of the stronger axillary odors (Leyden et al., 1981). Secretions were collected using 4-in. × 4-in. cotton pads worn in the axillae three times a week as in our previous studies (Preti et al., 1986; Zeng et al., 1991). Briefly, this protocol requires subjects to go without the use of deodorants, antiperspirants, deodorant soaps, or colognes in the axillae. They may wash once a day, only in the morning, with a nonfragranced soap (Ivory) during the collection protocol.

Preparation of Axillary Secretion Extract. A total of 24 pads were extracted to yield the axillary extract. Four pads from each subject were chosen. Extracts were prepared by extracting 12 pads at a time (two per subject) with double-distilled ethanol, as previously described (Cutler et al., 1986; Preti et al., 1987). After removal of the ethanol, pads were subsequently soaked with a mixture of 85:15 chloroform–methanol (15 ml/pad) for 1 hr and then squeezed. (Nanograde solvents were from Mallinkrodt, St. Louis, Missouri; these solvents contain few or no impurities by GC-MS analysis when 100–200 ml is concentrated to ≤ 50 μl.) The two 12-pad extracts yielded 300 ml and 310 ml total volumes, respectively. The two extracts were combined; 1 ml of saturated $NaHCO_3$ was added to this volume, and the extract was concentrated under vacuum to dryness. A 1.5-ml aliquot of doubly distilled water was employed to wash the flask and dissolve all dried solids. This aqueous solution was transferred to a 12-ml conical centrifuge tube in order to isolate the acidic compounds from the bases and neutral components.

Isolation of Acidic Components from Axillary Extract. The aqueous solution was extracted with 1.5 ml of Nanograde chloroform (Mallinkrodt) by vigorous

vortexing for 10 sec. The resulting two layers were separated by centrifugation in a clinical (IEF) centrifuge at top speed for 5 min. The bottom layer, containing the basic and neutral components, was transferred to a 10-ml vial by using a thinly drawn pipet. Two subsequent 1.5-ml aliquots of $CHCl_3$ were used to extract the aqueous layer, and the $CHCl_3$ layers were combined for 4.5 ml total. Fractionation of the basic and neutral compounds was done as described previously (Zeng et al., 1991), and the fractions were stored at $-10°C$ until they were concentrated and analyzed.

The aqueous layer remaining in the centrifuge tube was acidified by single drop additions of 2 N HCl until a pH of 2 was achieved. The mixture was extracted with $CHCl_3$ (1.5 ml $\times$ 2). The $CHCl_3$ layer was separated by centrifugation (as described above) and concentrated to 20 μl prior to analysis by GC-MS.

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis. A Finnigan 4510 GS-MS data system equipped with a split/splitless injector, a fused silica capillary column, and with capabilities for operation in both electron impact and chemical ionization modes was used for analyses. The columns employed were a 30-m $\times$ 0.32-mm-ID fused silica column with a 1.0-μm coating of Stabilwax (cross-bonded polyethylene glycol, Restek Inc., Bellefonte, Pennsylvania), or a 30-m $\times$ 0.32-mm-ID fused silica RTX-1 column with a 1.0-μm coating of cross-bonded methylsilicon (Restek Inc.).

The analysis conditions for the Stabilwax column were as follows: 60°C, 4 min, then 3°/min to 220°C; hold at 220°C for 1 hr. Analyses performed with the RTX-1 column used the following conditions: 60°C for 4 min, then 4°/min to 300°C; hold at 300°C for 20 min.

The mass spectrometer was interfaced with a Nova 4X computer that utilized the Super Incos software for data acquisition, analysis, and quantitation. The mass range employed during these analyses was typically m/z 40–400, with one scan per second. A typical run included 3600 scans. The data system also includes the NBS library of 42,000 compounds.

As in our previous studies (Zeng et al., 1991, 1992; Spielman et al., 1995), identifications were based on comparison of unknown spectra with those from synthetic or commercial standards. In addition, the relative chromatographic retention times of unknown and known standards were compared with a mixture of fatty acids ethyl esters (van den Dool and Kratz, 1963).

Collection of Apocrine Secretions. Four of the six female subjects who donated axillary secretions collected on pads agreed to donate apocrine secretions. The axillae was prepared for sampling and apocrine secretions stimulated by an intradermal injection of 1:10,000 adrenalin (in physiologic saline) as in our previous reports (Labows et al., 1979b; Zeng et al., 1992). The amount of secretion collected varied greatly with the individual subject (≤ 10 μl).

Separation of Apocrine Secretions in Aqueous and Organic Soluble Fractions. The apocrine secretions collected from each subject were combined by washing the vials with a total of 300 μl of doubly distilled water. Each of the collection vials was then washed with 400 μl of Nanograde chloroform. The chloroform and aqueous layers were combined and vigorously shaken for 10–15 sec. The mixture was centrifuged in a clinical (IEC) centrifuge for 5 min at top speed to form two layers. The bottom chloroform layer was removed in a thinly drawn pipet.

The remaining aqueous layer was extracted two more times with chloroform (2 × 400 μl) and subsequently separated by the same procedure as above. This procedure gave a volume of 1.2 ml of the chloroform solution and 300 μl of aqueous apocrine solution. Aliquots of 10 μl of the aqueous phase and 10 μl of the chloroform layer were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This is described below.

Hydrolysis of Apocrine Secretion Aqueous Phase. The aqueous apocrine solution was refluxed for 20 min with 1 ml, 5% aqueous sodium hydroxide under a stream of nitrogen. The mixture was allowed to cool to room temperature and acidified with 2 N HCl to pH 2. A slight axillary-like odor developed in the reaction mixture upon acidification. The acidified mixture was extracted with 3 × 150 μl chloroform. The combined chloroform extracts were concentrated to approximately 5 μl for organoleptic evaluation and GC-MS analysis.

Hydrolysis of Apocrine Secretion Organic Phase. The chloroform extract was concentrated to approximately 100 μl and refluxed for 20 min with 5% NaOH in 2 ml of a 1 : 1 solution of methanol–water under a stream of nitrogen; the same conditions were also used for the aqueous-phase hydrolysis. After removal of the solvent, the reaction mixture was acidified with 2 N HCl of a pH of 2; no odor was detected. This acidic solution was extracted with chloroform (3 × 0.5 ml) and concentrated to approximately 5 μl for organoleptic and GC-MS analyses.

Organoleptic Evaluation of Axillary Extracts and Hydrolyzed Apocrine Secretion Samples. Organoleptic evaluation was performed by a panel of three or four judges in a manner similar to that used previously for evaluation of chromatographic eluants (Zeng et al., 1991). Judges were presented in a blind manner with either fractions of axillary extracts or the acidified, hydrolyzed aqueous or organic portions of apocrine secretion and asked which had an odor that most resembled the axillary odor. A small (2- to 5-μl) aliquot of concentrated axillary extract on a filter-paper strip (in a sealed vial) was on hand for reference if requested by a judge.

SDS-PAGE of Apocrine Secretion Aqueous and Organic Soluble Fractions. SDS-PAGE was done according to Laemmli (1970). The stacking gel was 5% acrylamide and the separating gel was 15%. Samples (10 μl) of each fraction

were dried down to approximately 5 μl, then mixed with 5 μl of sample buffer containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, and 50 mM Tris HCl at pH 6.8 (Laemmli, 1970). After electrophoresis, the gels were stained with Coomassie blue.

RESULTS

Organoleptic evaluation of the concentrated neutral, base, and acidic components from the pad extract demonstrated that the acidic portion of the extract contained the characteristic axillary odor and most resembled the entire concentrated extract, a result analogous to that found with male samples (Zeng et al., 1991). Consequently, analyses by GC-MS were performed on the components in the acidic fraction. All the pads used in the extracts were collected during the presumed ovulatory portion of the menstrual cycle ($\pm$3 days of presumed ovulatory day) as judged by the subjects' basal body temperature charts and cycle length.

Figure 1 shows the reconstructed ion chromatogram (RIC) resulting from the analyses of the acidic components using the Stabilwax column. The part of the chromatogram shown in Figure 1 (bottom) is the area in which the C_6–C_{11} acids elute; these compounds have been shown to constitute the characteristic axillary odor in males. Both the identity of the lettered components and their presence in male extracts are listed in Table 1.

The top part of Figure 1 shows a mass chromatogram of m/z 60. This ion is formed by McLafferty rearrangement and is generally the most intense ion in the mass spectrum of aliphatic acids (with an available γ-hydrogen and unsubstituted α carbon) (Budzikiewicz et al., 1967). The large, regularly spaced maxima for peaks showing m/z 60 in Figure 1 suggest the presence of a series of *n*-aliphatic acids. Interpretation of the mass spectra of these chromatographic peaks when considered together with their relative retention times (ethyl ester units) (van den Dool and Kratz, 1963) led to the conclusion that these compounds were *n*-organic acids: hexanoic (peak A) through undecanoic acid (peak Gg).

The identity of all the peaks labeled in Figure 1 are shown in Table 1. This table also indicates which of these compounds were also found in the characteristic axillary odors from males (Zeng et al., 1991). Figure 2 shows the bottom portion of Figure 1 and the same portion of the chromatogram from the previously published combined male sample (Zeng et al., 1991) to show the relative differences between the male and female samples. Peak L in the bottom portion of Figure 2 is *E*-3M2H, and peak K in the top part of this figure is *E*-3M2H (male sample). Peaks H and G, bottom and top, respectively, are the *Z* isomer. Although *E*-3M2H was the major component in the combined male sample, this

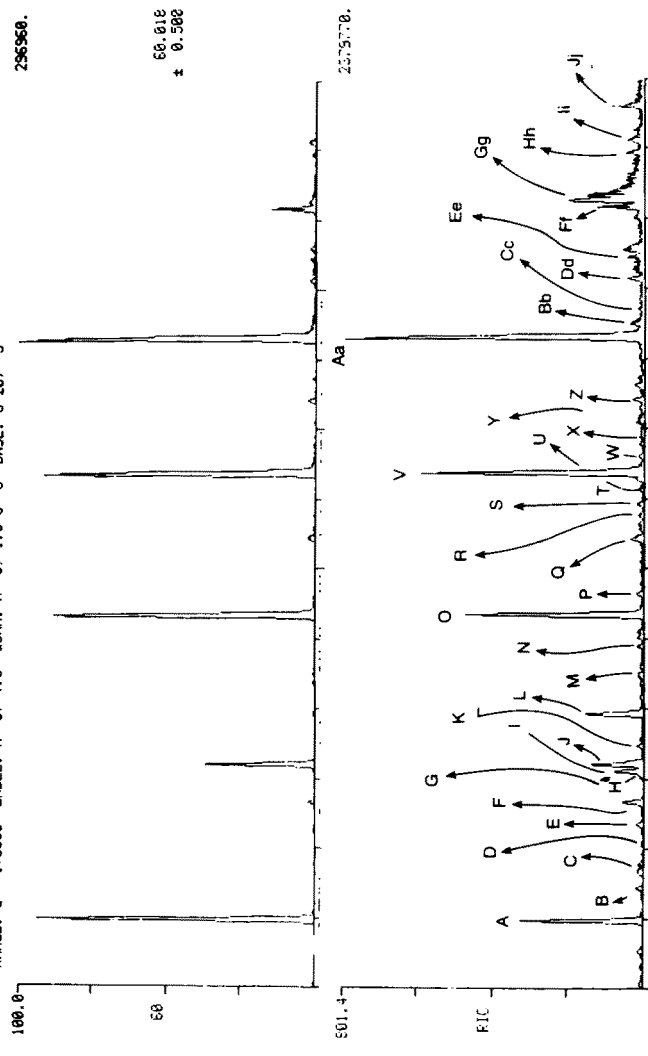

FIG. 1. The top part of the figure shows a plot of m/z 60 (mass chromatogram) in the area where the characteristic axillary odors elute. This ion is prominent in the mass spectra of aliphatic acids with an unsubstituted α-carbon and available γ-hydrogen as discussed in the text. This ion is the base ion for many normal-chain acids. The identity of the labeled peaks in the reconstructed ion chromatogram (bottom portion of the figure) may be found in Table 1, together with their relative retention tiumes.

TABLE 1. COMPOUNDS IDENTIFIED IN COMBINED FEMALE (F) AXILLARY EXTRACTS IN COMPARISON TO MALES

Peak	Compound	Molecular weight (kDa)	Retention time (ethyl ester units)	Present in combined female (F) male (M) extract
A	*n*-Hexanoic acid[G]	116	12.20	F/M
B	2-Methylhexanoic acid[G]	130	12.39*	F/M
C	3-Methylhexanoic acid[G]	130	12.55*	F/M
D	(*E*)-3-Methyl-2-pentenoic acid	**114**	**12.60***	**F only**
E	Dimethylsulfone[G] ($C_2H_6SO_2$)	94	12.63	F/M
F	γ-C_8-Lactone[G]	142	12.79	F/M
G	4-Ethylpentanoic acid[G]	130	12.97*	F/M
H	(*Z*)-3-Methyl-2-hexenoic acid[G]	128	13.10*	F/M
I	2-Ethylhexanoic acid	144	13.13	F/M
J	*n*-Heptanoic acid[G]	130	13.22*	F/M
K	2-Methylheptanoic acid[G]	144	13.36*	F/M
L	(*E*)-3-Methyl-2-hexenoic acid[G]	128	13.50*	F/M
M	Phenol	94	13.65	F/M
N	γ-C_9-Lactone[G]	156	13.91	F/M
O	*n*-Octanoic acid[G]	144	14.28*	F/M
P	2-Methyloctanoic acid[G]	158	14.41	F/M
Q	4-Ethylheptanoic acid[G]	158	14.81*	F/M
R	7-Octenoic acid[G]	142	14.95*	F/M
S	2-Piperidone[G]	**99**	**14.55***	**F only**
T	γ-C_{10}-Lactone[G]	170	15.01	F/M
U	*n*-Tetradecanol[G]	214	15.21	F/M
V	*n*-Nonanoic acid[G]	158	15.28	F/M
W	2-Methylnonanoic acid[G]	172	15.38	F/M
X	(*E*)-3-methyl-2-octenoic acid[G]	**156**	**15.45***	**F only**
Y	4-Ethyloctanoic acid[G] ("goat acid")	172	15.64*	F/M
Z	Unsaturated C_9 acid[T]	156	16.04*	F/M
Aa	*n*-Decanoic acid[G]	172	16.28	F/M
Bb	2-Methyldecanoic acid[T,G]	186	16.36	F/M
Cc	Unsaturated C_{10} acid[T]	170	16.46*	F/M
Dd	4-Ethylnonanoic acid[T,G]	186	16.69*	F/M
Ee	9-Decenoic acid[G]	170	16.90*	F/M
Ff	*n*-Hexadecanol	242	17.24	F/M
Gg	*n*-Undecanoic acid[G]	186	17.29	F/M
Hh	4-Ethyldecanoic acid[T,G]	200	17.66	F/M
Ii	10-Undecenoic acid[G]	184	17.76*	F/M
Jj	Benzoic acid[T]	122		

"The following symbols are used in this table: T = tentatively assigned by mass spectral data; G = correspondence of mass spectrum and relative chromatographic retention times with commercially available or synthetic sample. *Compounds judged to be important contributors to axillary odor, from data reported in male samples (Zeng et al., 1991).

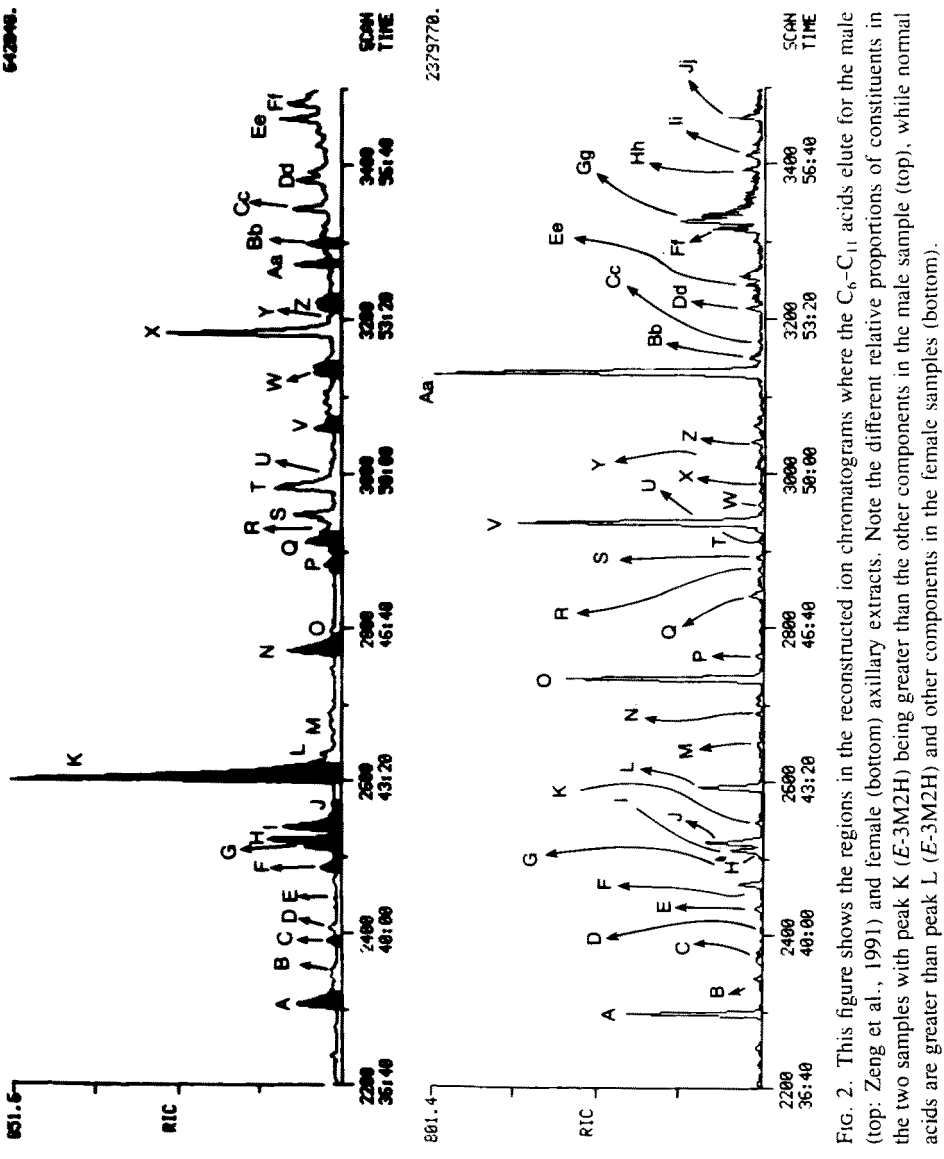

FIG. 2. This figure shows the regions in the reconstructed ion chromatograms where the C_6–C_{11} acids elute for the male (top: Zeng et al., 1991) and female (bottom) axillary extracts. Note the different relative proportions of constituents in the two samples with peak K (*E*-3M2H) being greater than the other components in the male sample (top), while normal acids are greater than peak L (*E*-3M2H) and other components in the female samples (bottom).

is not the case in the combined female sample. In the latter, the *n*-aliphatic acids are present in greater quantity than *E*-3M2H.

Comparison of injections of known amounts of 3M2H suggests ~150 ng/μl of *E*-3M2H is present in the female extract, while the male extract contained ~357 ng/μl of *E*-3M2H. In addition, the *E* to *Z* ratio in the combined female sample is 16:1, indicating a greater relative amount of the *E* vs. *Z* isomer in the female sample than previously reported in males, in which the ratio was 10:1 (Zeng et al., 1991).

The same series of γ-lactones reported in the combined male samples is present in the combined female sample. Although only C_8–C_{10}, γ-lactones are listed in Table 1, several lower members of the homologous series were also found eluting prior to the components in the area of the characteristic odor components.

Several other compounds whose structures were reported in male samples, and which are present here, included 7-octenoic acid (peak R) and two higher homologs (peaks Ee and Ii); goat acid (4-ethyl-octanoic acid, peak Y) and several homologs (peaks G, Q, Dd, and Hh); and 2-methylhexanoic acid (peak B) as well as four higher homologs (peaks K, P, W and Bb). Peak C was found to be 3-methyl-hexanoic acid.

Several subtle, qualitative differences between the male and female samples were seen, as noted in Table 1. Two homologs of the (*E*)-3-methyl-2-hexenoic acid are present in the female sample: 3-methyl-2-pentenoic (3M2P) and 3-methyl-2-octenoic (3M2O) acids. The relative retention times of both the lower and higher homologs matched with those of the synthetic *E* isomers we had in our possession (Zeng et al., 1992). The mass spectrum and relative retention time of peak S corresponded to 2-piperidone, a six-membered lactam.

To determine whether the aqueous soluble molecules from the apocrine secretions of females could be treated with base to yield 3M2H (Zeng et al., 1992), these secretions were separated into water-soluble and organic-soluble compounds as in our previous study (Zeng et al., 1992). Ten microliters of each fraction was saved for electrophoretic separation of putative proteins in each phase using SDS–polyacrylamide gels (Zeng et al., 1992). The rest of the aqueous and organic phases were treated with 5% NaOH to effect hydrolysis, and the acid components liberated by this procedure were isolated as in our previous study on male apocrine secretions (Zeng et al., 1992).

The reconstructed ion chromatograms (RICs) from the analyses of the aqueous and organic phase hydrolysates are shown in Figure 3A and B, respectively. Identity and/or data concerning the labeled peaks are shown in Table 2. Far greater amounts of *E* and *Z*-3M2H are seen in the hydrolysate of the aqueous phase than in the hydrolyzate of the organic phase molecules as indicated in Table 3. In addition, both the ratio of *E* to *Z* isomers in the aqueous hydrolyzate (~11.5:1) and the differences between the amount of *E* isomers in the aqueous

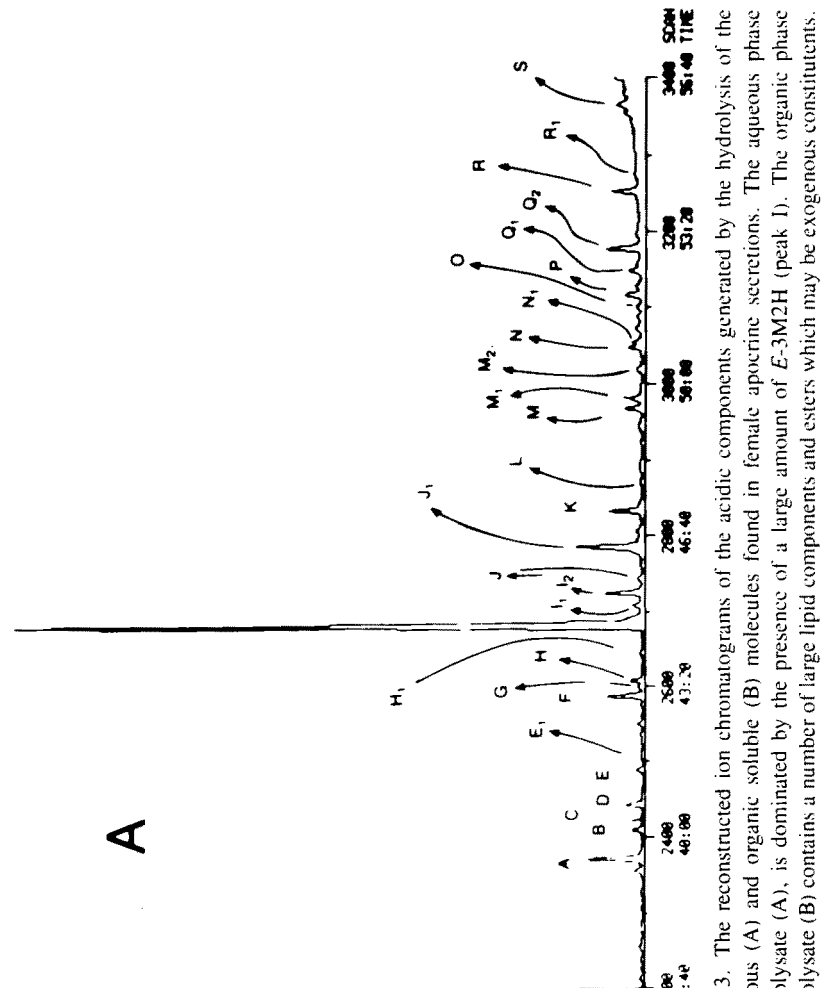

FIG. 3. The reconstructed ion chromatograms of the acidic components generated by the hydrolysis of the aqueous (A) and organic soluble (B) molecules found in female apocrine secretions. The aqueous phase hydrolysate (A), is dominated by the presence of a large amount of *E*-3M2H (peak I). The organic phase hydrolysate (B) contains a number of large lipid components and esters which may be exogenous constituents.

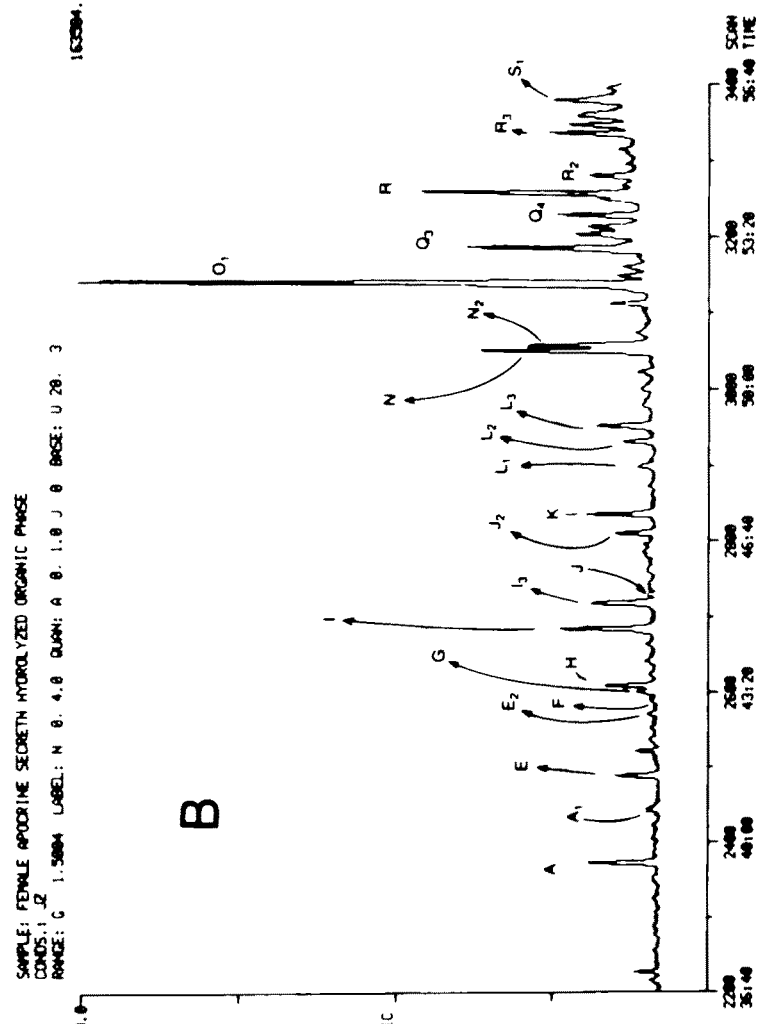

FIG. 3. Continued.

TABLE 2. COMPOUNDS IDENTIFIED IN HYDROLYZED FEMALE APOCRINE SECRETION: AQUEOUS PHASE MOLECULES OR ORGANIC PHASE MOLECULES[a]

Peak	Compound	Molecular Weight (kDa)	Found in aqueous phase (a) or organic phase (o) hydrolysate
A	*n*-Hexanoic acid[G]	116	a/o
A_1	Benzyl alcohol	108	o
B	Unknown[b]		a
C	2-Methylhexanoic acid[G]	130	a
D	Unknown[c] plus (*E*)-3-methyl-2-pentenoic acid[G]	114	a a
E	δ-Lactone[T,d]		a/o
E_1	Dimethylsulfone[G]	94	a
E_2	δ-Lactone[T,d]		o
F	(*Z*)-3-Methyl-2-hexenoic acid[G]	128	a/o
G	2-Ethyl-hexanoic acid[G]	144	a/o
H	*n*-Heptanoic acid[G]	130	a/o
H_1	2-Hexenoic acid[T,e]	114	a
I	(*E*)-3-Methyl-2-hexenoic acid[G]	128	a/o
I_1	C_7-unsaturated branched acid[T,f]	128	a
I_2	C_7-unsaturated branched acid[T,e]	128	a
I_3	δ-Lactone[T,d]		o
J	Phenol[G]	94	a
J_1	C_7-unsaturated branched acid[T,h]	128	a
J_2	Aliphatic alcohol[T,i]		o
K	*n*-Octanoic acid[G]	144	a/o
L	2-Methyl-octanoic acid[G]	158	a
L_1	Cyclohexane carboxylic acid[T,j]	128	o
L_2	Pentadecanoic acid methylester[T]	256	o
L_3	δ-Lactone[T,d]		o
M	7-Octenoic acid[G]	142	a
M_1	C_8-unsaturated acid[T,k]	142	a
M_2	C_8-unsaturated acid[T,l]	142	a
N	*n*-Nonanoic acid[G]	158	a/o
N_1	2-Methylnonanoic acid[G]	172	a
N_2	Unknown		o
O	(*E*)-3-Methyl-2-octenoic acid[G]	156	a
O_1	Palmitic acid methyl ester[G]	270	o
P	δ-Lactone[T,d]		o
Q_1	C_9-unsaturated acid[T,m]	156	a
Q_2	C_9-unsaturated acid[T,n]	156	a
Q_3	9-Hexadecenoic acid methylester	268	o
Q_4	Aliphatic alcohol[T,o]		o
R	*n*-Decanoic acid[G]	172	a/o
R_1	2-Methyldecanoic acid[G]	186	a
	Unsaturated C_{10} acid[T,p]	170	a

TABLE 2. Continued.

Peak	Compound	Molecular Weight (kDa)	Found in aqueous phase (a) or organic phase (o) hydrolysate
R_2	C_{17}-methyl branched methylester[T]	284	o
R_3	Heptadecanoic acid methylester[G]	284	o
S	Unknown long-chain alcohol[T]		o
S_1	Unsaturated C_{17} acid methylester[T]	282	o

[a]The following symbols are used in this table: T = tentatively assigned from mass spectral data; G = correspondence of mass spectrum and relative chromatographic retention times with commercially available or synthetic standards.

[b]Unknown seen only in aqueous phase hydrolysate: *m/z* (relative intensity) 45(20), 49(100), 51(31), 63(37), 77(2), 82(11), 84(4), 97(4), 112(42), 114(16): Chlorine containing?

[c]Unknown seen only in aqueous phase hydrolysate: *m/z* (rel. intensity) 41(45), 43(28), 55(100), 56(10), 56(10), 57(7), 59(10), 61(12), 69(38), 71(43), 81(12), 87(12), 97(41), 103(5), 105(4), 114(16), 115(12), 148(5): Sulfur containing?

[d]Proposed δ-lactones, seen only in organic phase, suggested by large ion an *m/z* 99 but few other ions suggestive of structure.

[e]Proposed 2-hexenoic acid, seen only in aqueous phase hydrolysate: *m/z* (rel. intensity) 41(48), 42(52), 43(22), 45(34), 55(35), 57(10), 60(11), 68(22), 71(8), 73(100), 74(12), 99(22), 114M$^+$(10).

[f]C_7 unsaturated branched acid, only in aqueous phase: *m/z* (rel. intensity) 41(90), 43(20), 45(10), 53(11), 55(54), 60(5), 67(35), 68(20), 69(100), 71(52), 82(22), 83(28), 85(6), 87(5), 95(6), 100(3), 113(10), 128(57). Isomeric with 3M2H?

[g]C_7 unsaturated branched acid, only in aqueous phase: *m/z* (rel. intensity) 41(82), 42(18), 43(30), 55(45), 57(12), 60(13), 67(30), 68(25), 69(100), 71(41), 82(42), 83(35), 95(8), 100(5), 113(12), 128(15). Isomeric with 3M2H?

[h]C_7 unsaturated branched acid, only in aqueous phase: *m/z* (rel. intensity) 41(30), 43(50), 55(11), 57(21), 71(100), 72(9), 73(4), 85(7), 100(1), 128(1).

[i]Aliphatic alcohol, only in organic phase hydrolysate: relative retention time suggests a C_{13} alcohol.

[j]Proposed cyclohexane carboxylic acid, seen only in the organic phase hydrolysate: *m/z* (rel. intensity) 41(55), 42(9), 43(11), 55(100), 56(29), 68(12), 69(29), 73(83), 83(50), 87(3), 99(9), 100(2), 110(4), 128(17).

[k]C_8 unsaturated acid, seen only in the aqueous phase: *m/z* (rel. intensity) 41(51), 43(37), 53(11), 55(100), 56(11), 57(11), 67(25), 68(10), 69(55), 71(19), 81(15), 82(62), 83(33), 96(18), 111(3), 124(9), 142(20). Isomeric with 7-octenoic acid?

[l]C_8 unsaturated acid, seen in the aqueous phase: *m/z* (rel. intensity) 41(71), 42(15), 43(54), 45(20), 54(16), 55(69), 56(33), 57(28), 67(28), 68(8), 69(18), 71(18), 73(9), 82(49), 83(21), 84(2), 85(100), 87(15), 96(16), 100(13), 111(2), 113(5), 124(19), 128(6), 142(1).

[m]C_9 unsaturated acid, seen in the aqueous phase: *m/z* (rel. intensity) 41(92), 42(25), 43(65), 45(31), 55(97), 57(29), 67(25), 68(35), 69(100), 71(32), 81(48), 83(38), 85(21), 96(65), 97(15), 114(42), 120(12), 138(1), 156(23).

[n]C_9 unsaturated acid, seen only in the aqueous phase. Mass spectrum reported previously (Zeng et al., 1992).

[o]Long-chain alcohol only in the organic phase, relative retention time suggests a C_{15} alcohol.

[p]C_{10} unsaturated acid, seen only in the aqueous phase: *m/z* (rel. intensity) 41(20), 43(100), 45(12), 55(20), 56(28), 57(6), 68(7), 69(13), 81(6), 96(5), 110(8), 114(10), 128(4), 152(3), 170(5).

TABLE 3. AMOUNTS OF 3-METHYL-2-HEXENOIC ACID LIBERATED FROM FEMALE APOCRINE SECRETION BY NaOH HYDROLYSIS

	Amount (ng/μl)	
	E isomer	*Z* isomer
Aqueous phase	244.7	21.2
Organic phase	1.4	0.01

phase (>200×) vs. the organic phase were almost identical to the ratios found in comparable male samples (Zeng et al., 1992).

As may be further observed in Figure 3A and Table 2, many of the same components seen in the combined female pad extract are apparently liberated by the hydrolysis procedure, albeit at different relative proportions than those in the male samples (Zeng et al., 1992). The organic soluble molecules contain many compounds that are lipophilic in nature, such as long-chain methyl esters and alcohols (peaks O_1, Q_3, Q_4, R_2, R_3, S_1) and may have been present in the apocrine secretions and extracted into the chloroform. A number of these compounds have been previously identified as compounds of exogenous origin in axillary area (Labows et al., 1979c).

Although 3M2H and normal C_6–C_{11} acids are present, their levels are far lower in the organic hydrolysate than in the aqueous phase hydrolysate (see Table 3). Further, the hydrolysate from the aqueous soluble molecules contain traces of *E*-3-methyl-2-pentenoic and *E*-3-methyl-2-octenoic acids (3M2O) as well as 7-octenoic acid. A number of the compounds found only in the aqueous phase appear to be acids whose molecular weights and mass spectral fragmentation patterns suggest that they are isomeric with 7-octenoic acid (M), 3M2H (I), and 3M2O (O): isomers of M are M_1 an M_2; isomers of 3M2H are I_1, I_2, and J_1; and isomers of O are Q_1 and Q_2. Further, the mass spectrum of Q_2 appears to be similar to that of an unknown C_9-unsaturated acid discussed in our previous studies on males (Zeng et al., 1991, 1992).

Other interesting compounds are the 2-hexenoic acid (tentatively identified from its mass spectrum) and peaks B and D found in aqueous phase hydrolyzate. The fragmentation of the latter two (see Table 2) suggest that they may be chlorine- (peak B) or sulfur-containing (D) compounds.

The results of the electrophoretic separation shown in Figure 4 were analogous to that found in males. The figure showing the gel on which the male apocrine secretion proteins were separated (lanes 2 and 3) [shown previously in Zeng et al. (1992)] was placed side-by-side with the female samples for com-

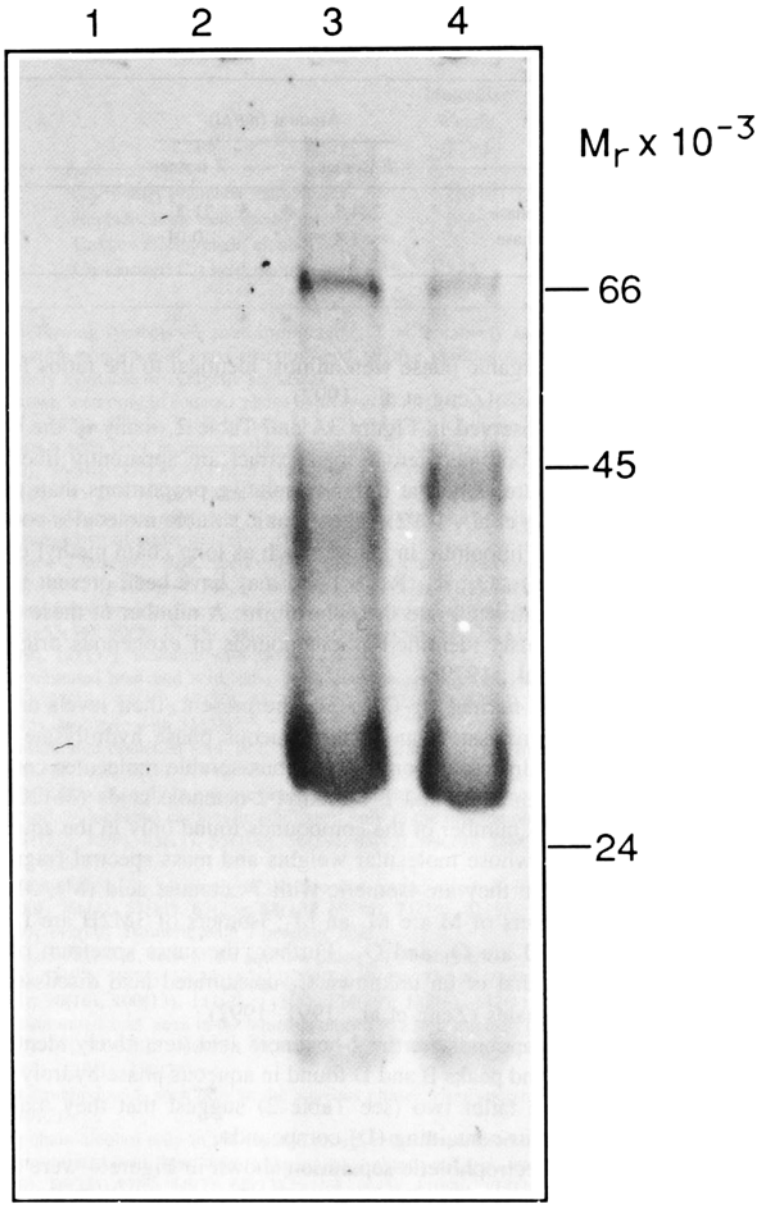

FIG. 4. The SDS-PAGE profile of the unhydrolyzed organic (lanes 1 and 2) and aqueous phases (lanes 3 and 4) from female (lanes 1 and 4, respectively) and male (lanes 2 and 3, respectively) apocrine secretions. The aqueous phase of both sexes display very similar profiles. Molecular weight markers were used to generate the scale to the right.

parison (lanes 1 and 4). The aqueous phase from female apocrine samples appears rich in proteins (lane 4), while the organic phase demonstrated no staining indicative of protein constituents (lane 1). The major protein bands in the aqueous phase are found at 26, 45, and 66 kDa. In male apocrine secretions, the proteins found at apparent molecular masses of 26 kDa and 45 kDa have been shown to carry 3M2H to the skin surface (Spielman et al., 1995).

The acid fraction of the combined pad extract as well as the apocrine secretion aqueous and organic phase hydolysates were examined for the presence of volatile steroids and/or steroid sulfates by GC-MS using a previously developed analysis protocole on a bonded methyl silicon column (Zeng et al., 1992; Preti et al., 1987). The combined pad extract and the aqueous phase hydrolysate contained androstenol (5α-androst-16-en-3α-ol) but no detectable amounts of androstenone (5α-androst-16-en-3-one). Neither of these volatile steroids was seen in the organic phase hydrolysate. The approximate levels of androstenol (i.e., when compared to injection of standards) were 3.5 ng/μl for the acid fraction from the combined pad extract and 0.81 ng/μl for the hydrolyzate from the aqueous soluble molecules from female apocrine secretions. Each of the samples analyzed contained detectable amounts (no quantitation attempted) of 17-oxo-5-androsten-3α-yl sulfate (dehydroepiandrosterone sulfate) and 17-oxo-5α-androstan-3α-yl sulfate (androsterone sulfate); these two sulfates were detected as their pyrolysis products (Labows et al., 1979b; Preti et al., 1987). In addition, each of the three samples contained the free steroids androsterone and dehydroepiandrosterone.

DISCUSSION

The results of our study demonstrate that men and women with similar axillary microflora are qualitatively similar in the array of volatiles produced. The characteristic axillary odor in the combined female axillary pads resides with the acidic compounds; the neutral/basic compounds possess little or no odor. The major components in both sexes were the same, the normal C_6–C_{11} acids and *E*-3M2H; however, as noted above, the relative proportions are reversed in the females' sample vs. the males'; in the latter, the *E*-3M2H was present in larger amounts than any of the normal C_6–C_{11} acids. The female sample also had a different ratio of *E*:*Z* isomers for 3M2H in the pad extracts; males have a 10:1 ratio, while a 16:1 ratio was seen in the female pad extract.

The combined pad extracts from both males and females did not employ internal standardization; therefore, the amounts of 3M2H and androstenol noted in the results are estimated, post-hoc, from comparison with injections of known amounts of standard compounds in the GC-MS system. The recovery of 3M2H and androstenol during extraction in combined pad samples is estimated to be 59% and 67%, respectively (Zeng et al., 1991; Preti et al., 1987).

Some subtle differences between males and females were noted in the constituents found in the combined pad extract acid fraction. For example, the female sample contained traces of *E*-3M2H and *Z*-3M2O and 2-piperidone. The latter compound is a six-membered ring lactam and is most likely not present as such in the pads, but may arise from a thermally induced cyclization of the compound δ-amino pentanoic acid (in the injector of the GC). We cannot explain why this compound was not seen in males.

In our previous study with the acid fraction from the combined male pad extract, we only determined that androstenol and androstenone were present via mass spectrometric evidence and retention times (Zeng et al., 1991). A re-examination of these data and comparison to standard injections of these steroids analyzed near the time frame this sample was run suggests that both compounds were present at an estimated level of ~0.5 ng/μl of extract. Consequently, the acid fraction from the combined female pad extract appears to have more androstenol than the acid fractions of the combined male pad extract; however, the female sample had no detectable amount of androstenone. It is likely that most of these steroidal molecules are extracted into the neutral components.

The female pads used in this study came from the presumed ovulatory portion of the menstrual cycle of each subject. Consequently, the relative proportions of the various components discussed here may differ in other parts of the menstrual cycle. Further, it is not known whether one donor contributed more than others to the levels of 3M2H, C_6–C_{11} acids, androstenol, and other compounds. This remains to be determined by the analyses of daily axillary samples from individual donors.

In contast to the female pad extract, the dominant component in the acids liberated from the aqueous soluble molecules was 3M2H, not normal C_6–C_{11} acids. This may reflect the individual contributions of one or more donors and/or possibly the time in the menstrual cycle. Because of the individual variability in the amount of apocrine secretion that could be collected, it was not possible to pool apocrine secretions from distinct times in the menstrual cycle; the results above represent the hydrolysis of a pooled sample from donors at different times in the menstrual cycle.

Another factor that may influence the relative proportions of compounds is the formation from axillary bacteria vs. liberation of these compounds by NaOH. The bacteria may add their own array of metabolites to the odor as it "matures" under the arm or release components from the apocrine secretions in a far different manner than does a base hydrolysis (Zeng et al., 1992).

The γ-lactones listed in Table 1 are minute components, as seen in Figure 1. They are most likely due to the metabolic action of lipophilic yeasts such as *Pityrosporum* species. We postulate this origin for γ-lactones, as we did for similar compounds in male axillary samples. These organisms are found in areas of human skin that contain large numbers of sebaceous glands, such as the

axillae and scalp (Leyden et al., 1991; Labows et al., 1979a). *Pityrosporum ovale* species isolated from the scalp have been shown to produce γ-lactones in the presence of human sebum; these lactones have a pleasant odor (Labows et al., 1979a).

Unlike the base-hydrolyzed male aporcine phase (Zeng et al., 1992), the comparable female samples studied here yielded androstenol after hydolysis. Whether this means that androstenol is secreted bound to a different, base-labile precursor or that there is more of the same precursor present in females than males and therefore has a greater probability of interacting with base and liberating the free steroid is not known.

Small amounts of C_6–C_{11} normal-chain acids and 3M2H were present in the organic phase hydolysate. This suggests that their precursors may have been present in minute quantity in the organic phase (slight solubility) because the separation of apocrine secretions into two phases may not be totally complete. Another possibility is that the volatile compounds were present at low levels (below olfactory threshold) in the apocrine secretion (Labows et al., 1979b; Gower and Ruparelia, 1993) prior to hydrolysis. As may be seen in Table 2, there are a number of lipophilic compounds (long-chain alcohols and δ-lactones) that were most likely present in the secretion at the times of collection (perhaps in the hair follicle near the skin surface) and partitioned in the organic phase and/or were formed during the hydrolysis reaction. Some compounds present in the organic phase, as noted above, such as methyl esters, may be present as artifacts from past use of cosmetic products (Labows et al., 1979c); however, we cannot explain why these esters remain after a base hydrolysis, except that the hydrolytic conditions may not have been harsh enough for total ester conversion to their respective acids.

The qualitative similarity in the bouquet of volatile organic acids that make up the characteristic female and male axillary odors suggest a similar origin and mechanism for odor production in males and females. In addition, experiments reported here, which utilized female apocrine secretions, demonstrate that: (1) the characteristic axillary odors can be released from the aqueous soluble molecules while only traces of 3M2H are present in the organic soluble molecules, (2) approximately 200× more 3M2H is liberated by hydrolysis of the aqueous phase molecules than the organic phase molecules, and (3) the electrophoretic profile of proteins in the aqueous soluble molecules from female and male apocrine secretion is qualitatively similar. Consequently, the water-soluble components of apocrine secretions carry 3M2H and other components of the characteristic axillary odor to the surface where they are liberated by the resident, axillary microflora, particularly the lipophilic diphtheroids. Since similar arrays of proteins are present in the female and male apocrine secretion aqueous phase, we may assume that individual proteins at 26 kDa and 45 kDa carry 3M2H out to skin surface, as has been found for males (Spielman et al., 1995). Further

support for this comes from preliminary immunohistochemical data, which reveal these two proteins in the apocrine glands of females and males (Spielman et al., 1994).

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REPRODUCTIVE BIOLOGY OF THE YELLOW-SPOTTED LONGICORN BEETLE, *Psacothea hilaris* (PASCOE) (COLEOPTERA: CERAMBYCIDAE). III. IDENTIFICATION OF CONTACT SEX PHEROMONE ON FEMALE BODY SURFACE

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Abstract—A major component of female contact sex pheromone of the yellow-spotted longicorn beetle, *Psacothea hilaris* (Pascoe), was isolated from the elytra and identified as (*Z*)-21-methyl-8-pentatriacontene. The synthetic compound released the typical mating behavior including holding, mounting, and abdominal bending in males, although its activity was considerably lower than the extract of female elytra when treated on a gelatin capsule as an artificial female model.

Key Words—Cuticular hydrocarbon, contact pheromone, sex pheromone, mating behavior, Coleoptera, Cerambycidae, *Psacothea hilaris*, (*Z*)-21-methyl-8-pentatriacontene.

INTRODUCTION

The importance of contact sex pheromone(s) in mating behavior of the yellow-spotted longicorn beetle, *Psacothea hilaris* (Pascoe) was demonstrated through analysis of the mating sequence (Fukaya and Honda, 1992). Adults were

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observed to mate promiscuously when they encountered potential mates while wandering or resting on host trees, including the mulberry, *Morus bombycis* (Yokoi, 1990; Iba, 1993). Typically, a male dashes toward a female, and immediately after antennal contact with her holds her body with his forelegs and mounts on her back. The male then adjusts his body axis to hers and tries to make parameral contact, which is necessary for successful copulation (Fukaya and Honda, 1992). This succession of events in the mating behavior of males is released by a contact pheromone on the female body surface (Fukaya and Honda, 1992). The sex pheromone consists of at least two components with different functions. One component is distributed mainly on female prothorax and induces dashing toward a female immediately after antennal contact to her. The other component, on her whole body, releases a series of mating behaviors from holding to abdominal bending (Fukaya and Honda, 1995). The present report deals with the chemical identification of the major component of the contact sex pheromone from female elytra, which releases male mating behaviors from holding to abdominal bending.

METHODS AND MATERIALS

Insects. The *Psacothea hilaris* beetles used for the bioassay were the progeny of beetles collected from the mulberry gardens in Ibaraki and Gunma prefectures in 1992 and 1993. The larvae and adults were individually reared on an artificial diet for silkworms (wet type Silkmate 2S, Nihon Nosan Kogyo Co. Ltd.) at 25°C and under a 15L:9D photoperiod regime (Fukaya and Honda, 1992). For the bioassay, reproductively mature males were selected from 14- to 60-day-old nonmated individuals by checking their response to intact females.

Bioassay. Pheromonal activity was evaluated according to the methods previously described by Fukaya and Honda (1992). A gelatin capsule (24 mm length, 8 mm diam., Japan Pharmacopoeia No. 00) was fixed on a filter paper disc (9 cm diam., Toyo No. 2) and coated with the test solution. After evaporation of the solvent, a treated capsule (or an intact female) was exposed to a male beetle, and the male response was observed for 5 min after encounter with the capsule (or female). Typical responses to the elytra extracts included holding the capsule, mounting the capsule, and abdominal bending (copulatory attempt) toward the capsule (Figure 6 below). Abdominal bending, the final step before copulation, was taken as a positive mating response in this study. Assays were conducted using 50 males within 3 hr before the beginning of scotophase under 25°C, 60% relative humidity, and ca. 300 lx.

Extraction. Extracts were obtained from the elytra of the females that were collected in mulberry fields in Gunma and Tochigi prefectures in 1993 and 1994. The elytra were removed from 180 females and dipped in 200 ml of ether for

24 hr. Extracts were filtered, dried over anhydrous Na_2SO_4, concentrated under reduced pressure, and the residue was dissolved with hexane. The extract solution was kept in a glass tube below −20°C until use.

Chromatography. The extract of elytra was chromatographed on 10 g of silica gel (Wakogel C-200, 200 mesh, Wako Pure Chemical Industries Ltd.) and eluted successively with hexane, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% diethyl ether in hexane; and diethyl ether (150 ml each). Active fractions were further fractionated by column chromatography on 1.5 g of silica gel impregnated with 10% $AgNO_3$ (200 mesh, Aldrich Chemical Company, Inc.). The column was washed successively with 20 ml each of hexane and 0.5%, 1%, 2%, and 5% ether in hexane.

Gas Chromatography (GC). GC analyses were conducted on a Hewlett-Packard (HP) 5890II gas chromatograph equipped with a cool on-column capillary injector and a flame ionization detector (FID). A fused silica capillary column (HP-1, 12 m × 0.2 mm ID, 0.33-μm film thickness; HP) was used for analyses. Injector temperature was programmed to equal the oven temperature plus 3°C. Injections were made at an initial oven temperature of 50°C. After 1 min the temperature program was started (20°C/min to 150°C and then 5°C/min to 310°C) and held at the final temperature for 20 min. Helium was used as the carrier gas.

Preparative Gas Chromatography. A large-diameter capillary column (HP-1, 5 m × 0.53 mm ID, 2.65-μm film thickness; HP) was connected to the cool on-column injector. The GC column effluent (30 ml/min) was split at the outlet end of the column to the transfer tube and the FID at a ratio of 5:1. The compounds eluted from the transfer tube were collected with a glass capillary (1 mm ID × 12.7 cm) at room temperature. Helium was used as the carrier gas.

Gas Chromatograph–Mass Spectrometer (GC–MS). GC–MS analyses were conducted with a JEOL SX-102A double-focusing magnetic sector mass spectrometer in the EI mode (70 eV, 170°C), which was interfaced with an HP5890II GC. The GC contained a 30-m × 0.25-mm-ID DB-1 capillary column (J&W) with a cool on-column injector. Injections were made at an initial oven temperature of 50°C. After 1 min the oven temperature was increased at 20°C/min to 120°C, and then at 5°C/min to 310°C, and held at the final temperature for 20 min.

Proton Nuclear Magnetic Resonance (PMR). PMR analyses were conducted with a JEOL model JMN-Eclipse 500-MHz Fourier-transform NMR spectrometer system. Data points (16,000) were collected with a 5.6-μsec pulse.

Microchemical Reactions. Microozonolysis was conducted according to Beroza and Bierl (1967). Oxygen containing ozone was blown into ca. 10 μl of hexane solution of the sample below −50°C through a glass capillary tube for 2 min. For GC–MS analysis of carbonyl products, 0.5 μl of the solution was

immediately injected. The remaining products were treated with a few milligrams of $NaBH_4$ in ca. 10 μl of ethanol for 17 hr at ca. 60°C to reduce compounds to the corresponding alcohol compounds. The alcohol products were purified with a short Florisil column (6% aqueous; 200 mg in a Pasteur pipet). The alcohol compounds were then treated with 25 μl of methanesulfonyl chloride in 25 μl of dimethylformamide in a small sealed glass tube filled with N_2 and kept for 16 hr at 65°C according to the procedure of Evans et al. (1968). The reaction mixture was then directly poured onto a short column containing 200 mg of Florisil (6% aqueous). The chloride derivatives were eluted with 1 ml of hexane.

Chemicals. The authentic compounds, (*Z*)-21-methyl-8-pentatriacontene (*Z*8-21Me-35:Hy) and its *E* isomer (*E*8-21Me-35:Hy), were synthesized as racemic mixtures. The procedure will be published elsewhere. As standard compounds for GC analyses, straight carbon-chain hydrocarbons were used; they were purchased from Sigma Chemical Co.

RESULTS

The crude extracts of female elytra [180 female equivalent (FE)] were fractionated with silica gel column chromatography. In the bioassay, the fractions eluted with hexane and 10% ether in hexane released the abdominal bending (AB) response to males as potent as the crude extract at 1 FE/capsule. The 90% ether in hexane fractions showed weak activity. The hexane fraction was further fractionated by column chromatography on 1.5 g of $AgNO_3$-impregnated silica gel. Activity was found in the fraction eluted with 0.5% ether in hexane.

When the 0.5% ether in hexane fraction was injected into the GC, one large peak, which accounted for ca. 60% of the total peak area, and several minor peaks were observed (Figure 1). Therefore, this 0.5% fraction was injected into the preparative GC and two fractions were obtained: one contained only one major peak (major fraction) and the other several minor peaks (minor fraction). Activity was observed only when the major fraction was applied on a gelatin capsule (Figure 2). In separate experiments, the average amount of major component was estimated to be 53.5 $\pm$ 27.8 μg/female (mean $\pm$ SD, $N = 7$) and for the minor components 32.3 $\pm$ 11.6 μg ($N = 7$).

The major fraction was injected into the GC–MS system (Figure 3). The EI mass spectra of the major compound gave an evident molecular ion at *m/z* 504, which indicated that this compound was a monounsaturated 36-carbon hydrocarbon. In separate GC analyses, however, retention values of the major compound, tetratriacontane, and hexatriacontane were 33.88 min, 32.64 min, and 34.89 min, respectively, which suggested that the major compound has an alkyl branch.

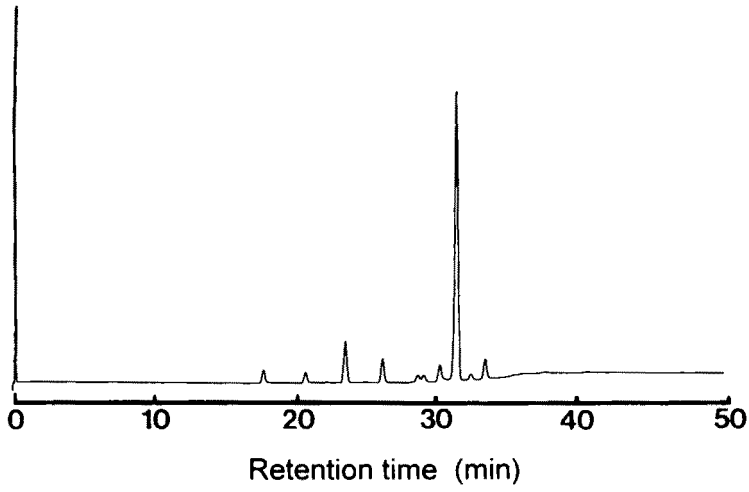

FIG. 1. Gas chromatogram of the 0.5% ether in hexane fraction eluted from $AgNO_2$-impregnated silica-gel column chromatography.

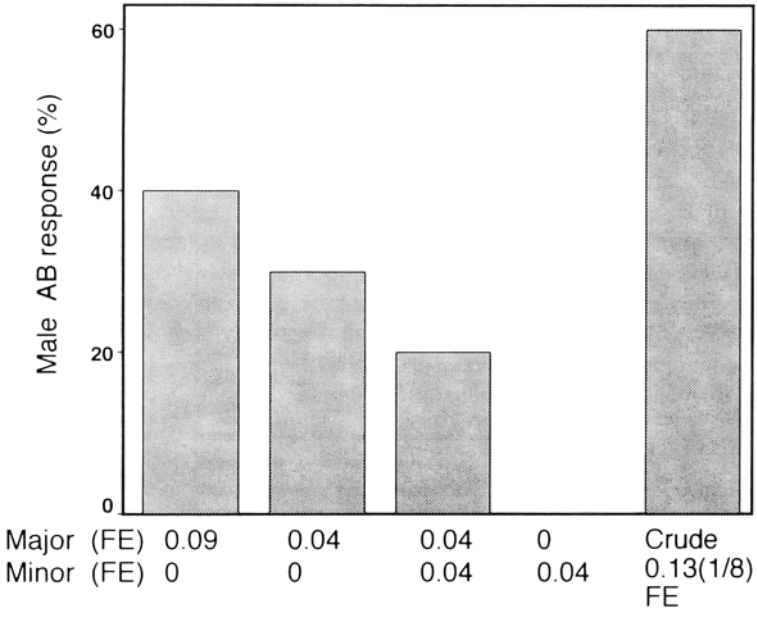

FIG. 2. Pheromonal activities of major and minor fractions from preparative GC and crude extract. Major fraction: 1 FE = 53.5 μg, minor fraction: 1 FE = 32.3 μg.

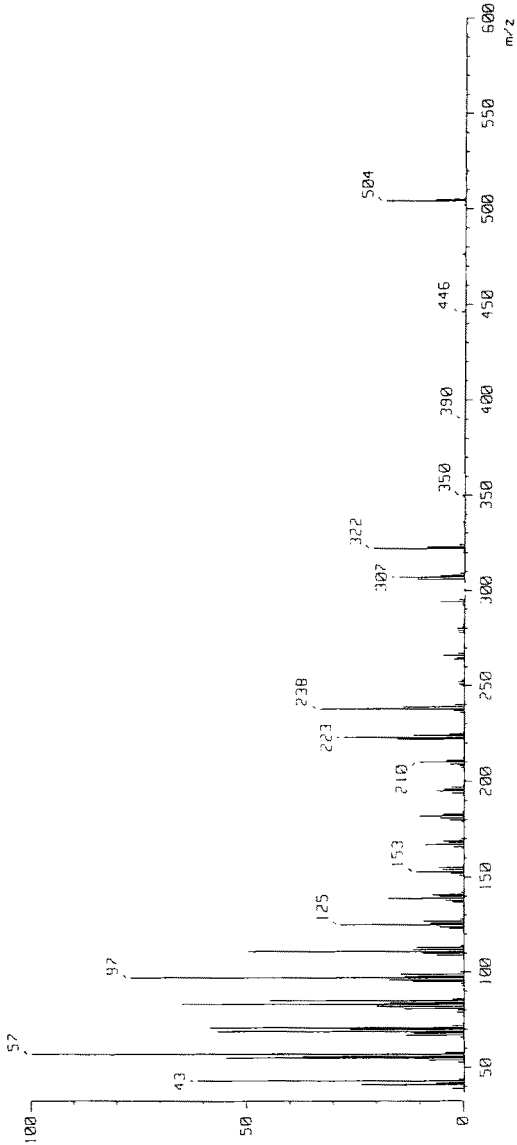

FIG. 3. EI mass spectrum of the major compound from preparative GC of 0.5% ether in hexane fraction.

Two large peaks and two small peaks were observed when ozonolysis products of the major fraction were injected into the GC–MS system. The large ones were octanal, whose mass spectrum and retention value were identical to those of authentic compound, and C_{28}-aldehyde, which was expected to contain an alkyl branch in the molecule. Minor products were decanal and C_{26}-aldehyde. These data indicated that the major and minor compounds in the major fraction have a double bond at the 8 and 10 positions, respectively.

To determine the position of the alkyl branch, the ozonolysis products were derivatized to chloride compounds. Mass spectrum of the major chloride derivative is shown in Figure 4. The molecular ion was evident at *m/z* 428 (intensity 1.32), which was accompanied by an M + 2 ion at *m/z* 430 (0.46). Diagnostic ions, which were generated by cleavage at the carbon branch, were observed at *m/z* 225 (48.16) and *m/z* 231 (29.01). The latter ion was accompanied by an M + 2 ion at *m/z* 233 (9.42), which indicated these ions contained one chlorine atom. The major chloride derivative was therefore considered to be 1-chloro-13-methylheptadocosane. This product, and the fact that octanal was detected as one of the major products of the ozonolysis reaction, indicate that the original major compound should be 21-methyl-8-pentatriacontene.

As minor derivatives, 1-chloro-11-methylpentadocosane and 1-chloro-13-methylpentadocosane were found. Based on similar interpretation, these two compounds indicated that the original parent molecules should be 21-methyl-10-pentatriacontene and 23-methyl-10-pentatriacontene, whose contents were 8.2% and 12.0% of the major compound, respectively.

The PMR spectrum (Figure 5) of ca. 5 mg of the 0.5% fraction indicated the presence of a monoolefin system. The signals of two olefin protons and four methylene protons neighboring the double bond were observed at 5.346 ppm and 2.012 ppm, respectively. These signals were also apparent in the PMR spectrum (800 scans) of ca. 5 μg of the major fraction collected from the preparative GC. In the NMR spectra of authentic *E* and *Z* isomers, chemical shifts (δ) for olefin protons were observed at 5.383 ppm and 5.349 ppm, respectively, and those for the methylene protons neighboring to the double bond were at 1.964 and 2.014 ppm, respectively. Thus, the geometric configuration was determined to be *Z*, and the pheromone component is (*Z*)-21-methyl-8-pentatriacontene (*Z*8–21Me–35 : Hy).

For evaluation of behavioral activity, synthetic Z8–21Me–35 : Hy was applied onto a gelatin capsule and male response was observed (Table 1). The synthetic compound elicited no dash behavior and released AB behavior for 62% of males at 107 μg/capsule (2 FE/capsule) (Figure 6). The female elytra extract elicited 94% of male AB at 1 FE/capsule, although the synthetic compound did not release the dash behavior to males. Therefore, the major sex pheromone component on the female elytra was identified as Z8–21Me–35 : Hy.

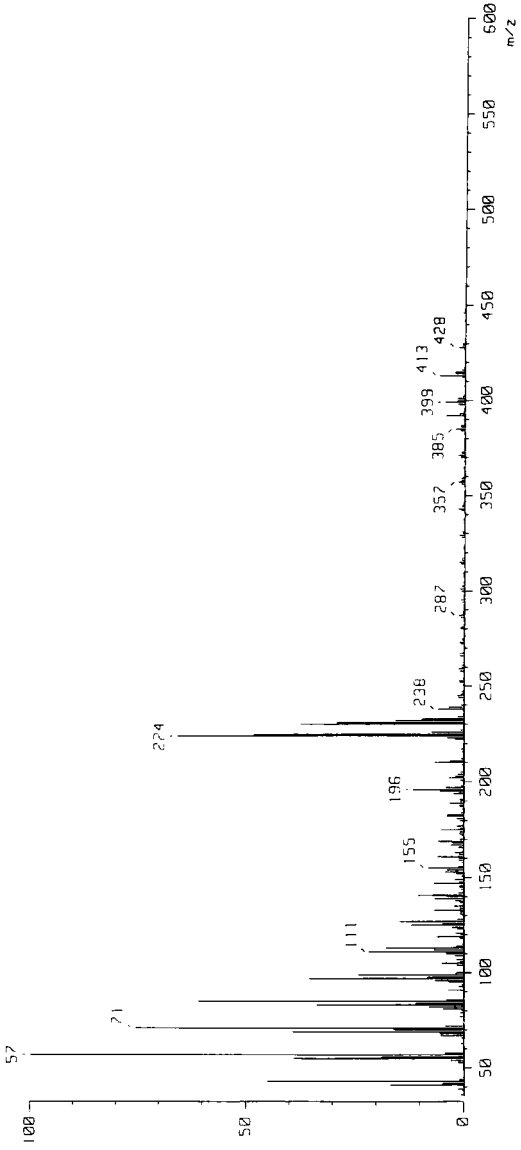

FIG. 4. EI mass spectrum of the major chloride derivative of ozonolysis products.

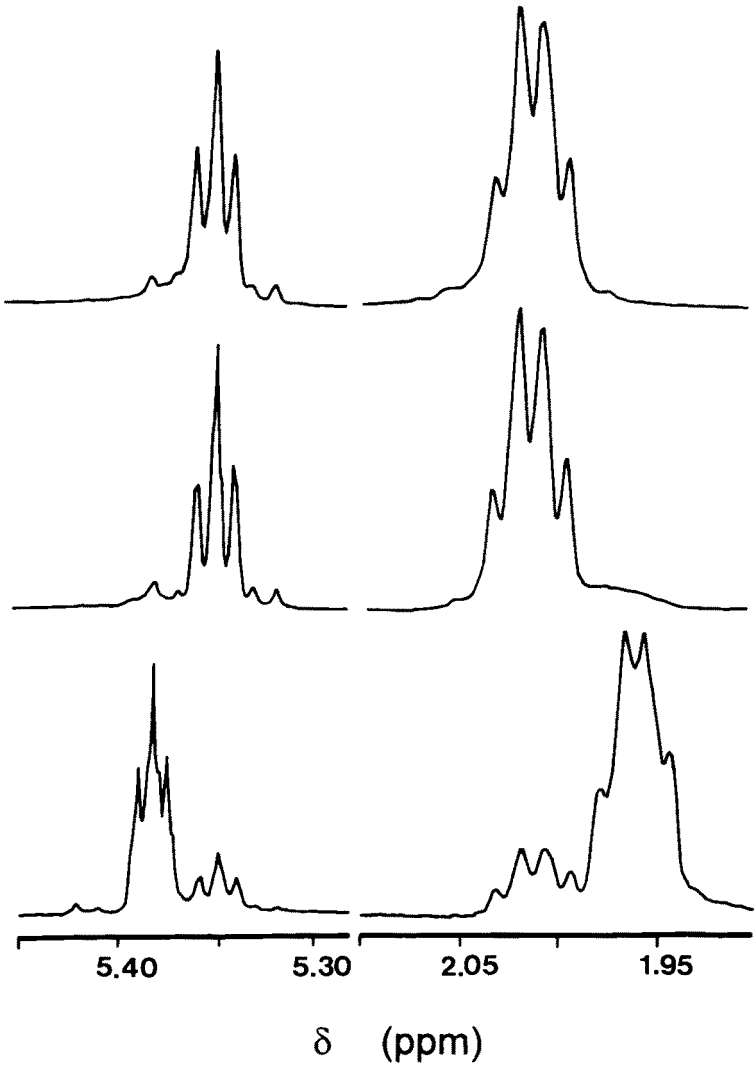

FIG. 5. PMR spectra of defined proton's and methylene proton's neighboring double bonds in the 0.5% fraction (top), synthetic (*Z*)-21-methyl-8-pentatriacontene (middle), and mixture of *Z* and *E* isomers of 21-methyl-8-pentatriacontene (bottom).

TABLE 1. MALE RESPONSES TO GELATIN CAPSULES TREATED WITH SYNTHETIC (Z)-21-METHYL-8-PENTATRIACONTENE (Z8-21Me-35:Hy).

Z8-21Me-35:Hy	Male response (%)[a]
27 μg (0.5 FE)	24 (13–38)
54 μg (1 FE)	36 (23–50)
107 μg (2 FE)	62 (47–75)
414 μg (4 FE)	70 (56–82)
Solvent (10 μl hexane)	0 (0–7)
Female elytra extract (1FE)	94 (83–98)
Intact female	100 (93–100)

[a]Values in the parentheses are 95% confidence intervals for binomial distribution ($N = 50$).

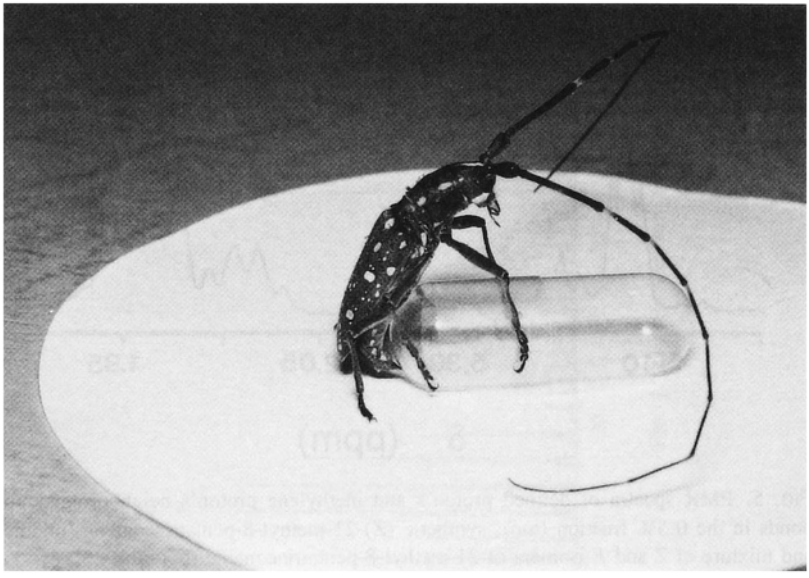

FIG. 6. Male abdominal bending response to a gelatin capsule covered with synthetic (Z)-21-methyl-8-pentatriacontene.

DISCUSSION

In the present study, we successfully identified one of the pheromone components from the female elytra as (Z)-21-methyl-8-pentatriacontene (Z8–21Me–35 : Hy). This compound released typical mating behavior in males from holding to abdominal bending. This is the first identification of a contact pheromone component of the female in cerambycids. Pheromones of several cerambycids were found to be perceived by contact or only at close range (Kuboki, et al., 1985, Wang et al., 1991, Fauziah et al., 1992), but none has been chemically identified. Kim et al. (1993) found hydrocarbon compounds in the female sex pheromone fraction of *Semanotus japonicus*. In cerambycids, sex attractants were also identified from a male (Iwabuchi, 1982, Sakai et al., 1984) and a female (Leal et al., 1994).

The activity of Z8–21Me-35 : Hy was, however, considerably lower than that of female extract (Table 1). In the present experiment, weak activity was eluted with 90% ether in hexane after the elution of major activity from silica gel column chromatography.

The synthetic Z8–21Me–35 : Hy released holding to abdominal bending behavior but not dash behavior. In a previous paper, at least two pheromone components with different functions were demonstrated: one induces dash behavior and the other elicits holding and abdominal bending (Fukaya and Honda, 1995). The major component, which showed the latter activity, was identified in the present study, but the identification of the compound(s) responsible for dash behavior remains to be conducted.

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ISOLATION, IDENTIFICATION, AND SYNTHESIS OF SEX PHEROMONE COMPONENTS OF FEMALE TEA CLUSTER CATERPILLAR, *Andraca bipunctata* WALKER (LEPIDOPTERA: BOMBYCIDAE) IN TAIWAN

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Abstract—Octadecanal (18:Ald), (*E*)-11-octadecenal (*E*11-18:Ald), (*E*)-14-octadecenal (*E*14-18:Ald) and (*E*,*E*)-11,14-octadecadienal (*E*11,*E*14-18:Ald) were isolated and identified as major components from the pheromone glands of the tea cluster caterpillar, *Andraca bipunctata*, in Taiwan by analyzing the mass spectra of gland components and their DMDS adducts. GC retention times and mass spectra of the components were in agreement with those of authentic synthetic compounds. The average amount of 18:Ald, *E*11-18:Ald, *E*14-18:Ald and *E*11,*E*14-18:Ald per female gland (1 to 3 days old) was 121 ± 76, 50 ± 20, 187 ± 75, and 237 ± 110 ng, respectively, in a ratio of 20:8:31:41. Synthetic *E*11,*E*14-18:Ald caught more males than each of the other three components or blank control in field trapping tests. *E*11,*E*14-18:Ald is reported as an insect sex pheromone for the first time. Male antenna responded to *E*11,*E*14-18:Ald strongly in an EAG analysis. Furthermore, 4 hr after the injection of PBAN (pheromone biosynthetic activating neuropeptide) into decapitated female moths (2 days old), the percentage of the *E*11,*E*14-C18 Ald in the gland extract increased from 0% to 75.5%, which was also significantly more than that of unligated and uninjected control at 55.1%. All these data indicated that *E*11,*E*14-18:Ald is the sex pheromone of the *Andraca bipunctata* in Taiwan.

Key Words—Decapitated female moth, octadecanal, (*E*)-11-octadecenal, (*E*)-14-octadecenal, (*E*,*E*)-11,14-octadecadienal.

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INTRODUCTION

The tea cluster caterpillar, *Andraca bipunctata*, distributed mostly in Sumatra, Java, India, southern China and Taiwan, causes severe damage to tea plants, often resulting in abandoning of whole tea plantations. Current method of controlling this pest with pesticides is ineffective (Banerjee, 1982; Tseng, 1994). In previous work, we demonstrated that the use of the female sex pheromone of the smaller tea tortrix moth, *Adoxophyes* sp., for monitoring population and mating disruption was very successful in tea plantations in Taiwan (Kou et al., 1990; Kou and Chow, 1991; Tsai and Chow, 1992). It is contemplated that the same strategy may be applicable to the control of the tea cluster caterpillar. The sex pheromone of the tea cluster caterpillar, *Andraca bipunctata*, however, has not been described. To facilitate possible future utilization, we characterized and identified the chemical constituents of the sex pheromone gland of the tea cluster caterpillar by analyzing the mass spectra of components in the glandular extracts and their DMDS adducts. The double-bond configuration was determined by comparing the natural products with authentic synthetic compounds on several GC columns. The effectiveness of each of the synthetic compounds was tested by the EAG method. Synthetic compounds or their mixtures were used to catch males in field-trapping tests. Results showed that *E*11,*E*14-18:Ald, or mixtures that contained *E*11,*E*14-18:Ald effectively caught more males than the blank control under field conditions. We concluded that (*E*,*E*)-11,14-octadecadienal is the sex attractant of *A. bipunctata* in Taiwan.

Previously sex pheromone has been identified for only two species in the family Bombycidae, *Bombyx mori* (Linnaeus) and *B. mandarina* Moore (Butenandt et al., 1959; Kuwahara and Yen, 1979; Mayer and McLaughlin, 1991). Both species use (*E*,*Z*)-10,12-hexadecadien-1-ol (bombykol) as the principal component of their sex pheromone. To our knowledge, this is the third member of Bombycidae whose pheromone has been identified. It is also the first identification of (*E*,*E*)-11,14-octadecadienal as an insect sex pheromone.

METHODS AND MATERIALS

Insects. The experimental colonies were set up with pupae supplied by the Taiwan Tea Experiment Station, Hsinchu, Taiwan. Hatched larvae were reared on fresh tea leaves. Male and female pupae were held in separate rooms at 20°C under a 14L:10D regime.

Preparation of Pheromone Gland Extracts. Ovipositors from 1- to 3-day-old virgin females were excised 2 hr into the scotophase and immersed in 10 μl hexane per gland for 2 min. The extracts were stored at 4°C until bioassay and structure analysis (Kou and Chow, 1991).

Derivatization. Dimethyldisulfide (DMDS) derivatives of monounsaturated and diunsaturated compounds were prepared as described previously (Buser et al., 1983; Vincenti et al., 1987).

Chemical Analysis. Gas Chromatography (GC) of the extract was performed on a Varian 3700 GC equipped with a flame ionization detector. Three different capillary columns were used: a 30-m × 0.25-mm-ID fused silica capillary column of DB-Wax phase, a 30-m × 0.25-mm fused silica capillary column of DB-23 phase, and a 30-m × 0.25-mm fused silica capillary column of DB-225 phase. Chromatographic conditions included nitrogen carrier at 0.5 kg/cm^2, isothermal column temperature at 200°C for the DB-Wax column; a temperature program from 100°C to 250°C at 5°C/min for the DB-23 column, and a program from 100°C to 220°C at 4°C/min for the DB-225 column.

Gas chromatographic–mass spectrometric (GC-MS) analysis was conducted using a Finnigan MAT Incos 50 spectrometer. Samples were introduced through a 30-m × 0.25-mm DB-23 capillary column programmed from 80° to 250°C at 5°C/min and held at 250°C for 5 min. Helium was used as the carrier gas. For the analysis of the DMDS adducts of the unsaturated compounds a 30-m × 0.25-mm DB-5 capillary column was used and the temperature was programmed from 150°C to 320°C at 1°C/min. Electron impact (EI) mass spectra were collected at 70 eV with the separator at 250°C and the source at 180°C.

Chemical Synthesis: (*E*)-11-Octadecenol was obtained from NU-Chek-Prep. Inc. Elysian, Minnesota. (*E*)-2-Hexenol, (*Z*)-2-hexenol, 1-pentyne, and 1-octyne were purchased from Tokyo Chemical Industry Co. Pyridinium dichromate (PDC), 10-bromodecanol, and 11-bromoundecanol were obtained from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin). 7-Dodecynol was purchased from Lancaster Synthesis Ltd. (Lancashire, United Kingdom). Octadecanol and dimethyl disulfide were obtained from Merck Chemical Company.

Octadecanal and (*E*)-11-octadecenal were prepared from octadecanol and (*E*)-11-octadecenol, respectively, using PDC as an oxidizing reagent according to Corey and Schmidt (1979). (*Z*)-11-Octadecenal, (*Z*)-14-octadecenal, (*E*)-14-octadecenal, (*E,E*)-11,14-octadecadienal, and (*Z,E*)-11,14-octadecadienal were synthesized according to the procedures outlined in Figure 3 below. (*E*)-2-Bromohexene was prepared from (*E*)-2-hexenol by reacting with HBr vapor. For the synthesis of (*Z,Z*)-11,14-octadecadienal, the same strategy was used in the preparation of (*Z,E*)- and (*E,E*)-octadecadienal, except that (*Z*)-2-hexenol was used as starting material.

Pheromone Titer Determination. The extracts were prepared as described above and subsequently analyzed with the external standard method of quantitative analysis. Pheromone biosynthesis activating neuropeptide (PBAN), a 33-amino acid peptide, was obtained from Dr. A. Raina (USDA INHL, Bldg 306, Barc-East, Beltsville, Maryland 20705) as a gift. For PBAN analysis, virgin female moths were ligated between the head and thorax and decapitated. Forty-

eight hours after decapitation, 5 pmol of PBAN was injected into the abdomen, and 4 hr later the sex pheromone gland was excised and extracted with hexane. Saline was injected into the abdomen of controls. The extract was stored at 4°C for later analysis. Quantitative analysis was performed by the external standard method as before.

EAG Analysis. Chemicals of different dosage were loaded in a 5-ml glass syringe and puffed over an antennae. The EAG response from the chemicals was compared to the response of the antennae to an air blank. Each chemical was tested using EAG with at least six different antennae. The EAG response was preamplified (100× or 1000×) and recorded with a V1000 video, 4035 digital storage oscilloscope and ES1000 recorder (Gould Co. Ltd.) (Yang et al., 1992).

Field Testing. Field testing of the synthetic chemical formulation was carried out first for released males in a tea plantation in Taiwan Tea Experiment Station, Puehsin, Hsinchu, Taiwan, during November 1994, and subsequently in a local open tea plantation near Academia Sinica for wild males in April and May 1995. Synthetic chemicals were used separately and as a mixture. A total of 0.1 mg synthetic compounds was dissolved in hexane and loaded in plastic tubes, 5 cm × 1 mm ID. The plastic tube was suspended on the inside at the top of a wing-shaped sticky trap (Kou et al., 1992). Blank traps and traps baited with two female extracts were used for comparison. Traps were placed 10 m apart and 1.5 m above ground. For the capture of released males, a total of 120 laboratory-reared 1- to 3-day-old unmated males were released. Each trap was checked every afternoon for eight days. For the wild male field tests, traps were checked every week.

RESULTS AND DISCUSSION

Chemical Analysis. GC analysis showed a total of four major peaks, labeled A–D, in the chromatogram of a gland extract (Figure 1). Component A, with a molecular weight of 268, had the same mass spectrum as octadecanal (Table 1). Components B and C, both having a molecular weight of 266, had virtually identical mass spectra, with only a slight difference in relative intensities. The mass fragmentation patterns suggested isomeric monounsaturated octadecenals. Component D had a molecular weight of 264, and the mass spectral fragmentation pattern suggested an octadecadienal.

Synthetic octadecanal matched component A from gland extract in retention times on three GC columns and by mass spectrometry (Table 2), and we concluded that component A is octadecanal (Figure 1). The double-bond position of the monounsaturated aldehydes, components B and C, were inferred by the DMDS derivatization method, which was previously used for the determination

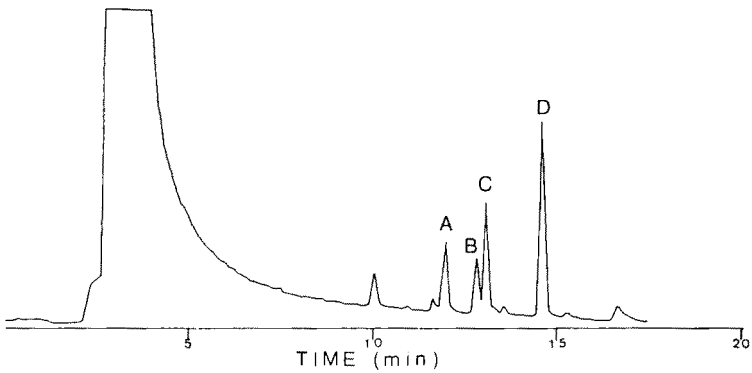


FIG. 1. Gas chromatogram of female sex pheromone gland crude extract. The gas chromatography was performed on a 30-m × 0.25-mm DB-Wax column and the oven temperature was isothermal at 200°C.

TABLE 1. MASS SPECTRAL DATA, AVERAGE QUANTITIES, AND PERCENTAGE OF FOUR COMPOUNDS IDENTIFIED FROM SEX PHEROMONE GLAND OF 1- TO 3-DAY-OLD *A. bipunctata* VIRGIN FEMALES (N = 22)

Compound	Mass spectral data, *m/z* (intensity, %)	Mean quantity (ng/female) (mean ± SD)	Percentage (mean ± SD)
18:Ald (A)	250(M^+-18, 4), 222(5), 194(4), 166(2), 138(10), 124(15), 110(18), 96(63), 82(90), 57(100)	121 ± 76	20 ± 3
*E*11-18:Ald (B)	266(M^+, 4), 248(18), 166(8), 135(20), 121(28), 111(32), 95(53), 83(60), 69(68), 55(100)	50 ± 20	8 ± 1
*E*14-18:Ald (C)	266(M^+, 4), 248(10), 166(4), 135(15), 121(20), 111(22), 95(43), 83(45), 69(49), 55(100)	187 ± 75	31 ± 7
*E*11,*E*14-18:Ald (D)	264(M^+, 3), 219(2), 165(1), 151(3), 123(3), 109(18), 95(33), 81(73), 67(100), 55(45)	237 ± 110	41 ± 4

TABLE 2. RETENTION TIMES OF 18:Ald, *E*11-18:Ald, *E*14-18:Ald, *E*11,*E*14-18:Ald, *Z*11-18:Ald, *Z*14-18:Ald, *Z*11,*E*14-18:Ald, *E*11,*Z*14-18:Ald, *Z*11,*Z*14-18:Ald AND PHEROMONE GLAND EXTRACT OF FEMALE *A. bipunctata* ON THREE GAS CHROMATOGRAPHIC COLUMNS

Compound	Source	Retention time (min)		
		DB-Wax	DB-23	DB-225
18:Ald	Natural	11.73	19.98	29.30
	Synthetic	11.79	20.01	29.344
*E*11-18:Ald	Natural	12.63	20.32	29.734
	Synthetic	12.67	20.34	29.760
*E*14-18:Ald	Natural	12.89	20.46	29.875
	Synthetic	12.93	20.48	29.903
*E*11,*E*14-18:Ald	Natural	14.39	20.94	30.483
	Synthetic	14.42	20.98	30.538
*Z*11-18:Ald	Synthetic		20.73	
*Z*14-18:Ald	Synthetic		20.89	
*Z*11,*E*14-18:Ald	Synthetic		21.16	
*E*11,*Z*14-18:Ald	Synthetic		21.34	
*Z*11,*Z*14-18:Ald	Synthetic		21.73	

of the double-bond position in monounsaturated acetates (Buser et al., 1983). DMDS adducts of the aldehydes were prepared, and the relevant mass spectral data are shown in Table 3. The mass spectrum of the DMDS derivative of component B showed a molecular ion at *m/z* 360 and two intense ions at *m/z* 145 and 215, presumably arising from a cleavage between the vicinal thiomethyl groups. Two possible aldehydes could yield the observed ions. The parent compound may be 11-octadecenal and the ion at *m/z* 145 could represent $CH_3-(CH_2)_5-CH=S^+CH_3$, which arises from the distal end of the molecule, whereas the ion *m/z* 215 would represent the functional-group-bearing end of the molecule (Figure 2A). Alternatively, the parent compound may be 6-octadecenal, with the ion at *m/z* 215 representing $CH_3-(CH_2)_{10}-CH=S^+CH_3$, arising from the distal end of the molecule, and the ion *m/z* 145 representing the functional-group-bearing end of the molecule. In order to distinguish between 11- and 6-octadecenal, the compounds in the gland extract were reduced to alcohols by $LiAlH_4$ and then derivatized to the DMDS adduct. The mass spectrum of the DMDS adduct of the alcohol of component B (Table 3) showed that the major fragments are at *m/z* 217 and 145, and we concluded that the component B is 11-octadecenal. The DMDS adduct of 6-octadecenol would give major fragments at *m/z* 147 and 215. Both (*E*)-11- and (*Z*)-11-octadecenal

TABLE 3. MASS SPECTRAL DATA OF DMDS DERIVATIVES OF MONOUNSATURATED AND DIUNSATURATED ALDEHYDES AND ALCOHOLS

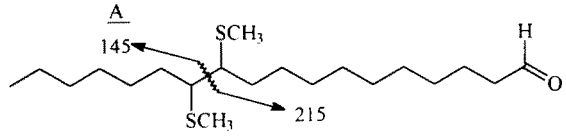
Compound	Mass spectral data of the DMDS derivatives (m/z, %)
*E*11-18:Ald (B)	360(M^+, 10), 243(12), 215(66), 145(95), 97(38), 81(33), 67(50), 61(85), 55(100)
*E*14-18:Ald (C)	360(M^+, 10) 257(72), 109(20), 103(60), 95(30), 81(30), 67(30), 61(100), 55(68)
*E*11-18:Alc	362(M^+, 0.25), 217(14), 145(21), 109(7), 95(17), 85(24), 71(34), 61(29), 57(91), 55(63)
*E*14-18:Alc	362(M^+, 0.36), 259(18), 109(8), 103(37), 95(16), 81(20), 67(24), 61(67), 55(61)
*E*11,*E*14-18:Ald[a] (D)	390(M^+, 3), 342(9-16), 239(48-100), 215(26-35), 175(20), 127(100), 103(19-38), 55(37-53)
*E*11,*E*14-18:Alc[a]	392(M^+, 2), 344(5-8), 241(12), 217(16-24), 175(10-24), 127(48-100), 103(19), 55(45-65)

[a]The ranges of abundance are due to the presence of chromatographically separated DMDS adduct isomers with slight differences in their mass spectra.

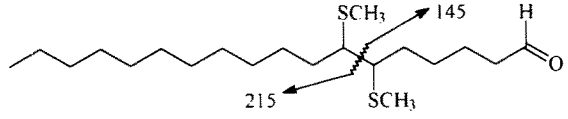
(Figure 3A) were synthesized and retention times on the DB-23 column were compared (Table 2). (*E*)-11-Octadecenal cochromatographed with component B of the gland extract, so it was concluded that component B is (*E*)-11-octadecenal.

Similar analysis of the mass spectrum of the DMDS derivative of component C (Table 3) revealed that the parent compound might be 14-octadecenal or 3-octadecenal (Figure 2B). Component C was reduced to the alcohol by $LiAlH_4$, as above, and the DMDS adduct of the corresponding alcohol was prepared and analyzed by GC-MS. The mass spectrum (Table 3) showed that the major fragments were at *m/z* 259 and 103, suggesting that the component C is 14-octadecenal, since the DMDS adduct of 3-octadecenol will give major fragments at *m/z* 105 and 257. Both *E* and *Z* isomers of 14-octadecenal were synthesized (Figure 3B) and compared with compound C on different GC columns (Table 2). The results indicated that (*E*)-14-octadecenal cochromatographed with component C of the gland extract and component C was identified as (*E*)-14-octadecenal.

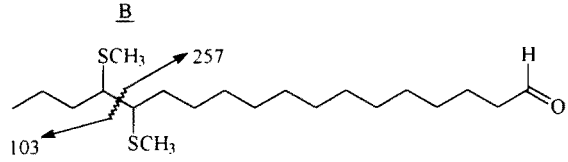
The diunsaturated octadecadienal was derivatized according to the procedure described by Vincenti et al. (1987). GC-MS analysis of the DMDS adduct showed fragments at *m/z* 215 and 103 (Table 3). From this fragmentation pattern, component D in the gland extract could be 11,14-octadecadienal or 3,6-octadecadienal (Figure 2C). Component D in the gland extract was reduced to an alcohol by $LiAlH_4$ and derivatized to its DMDS adduct. GC-MS analysis of



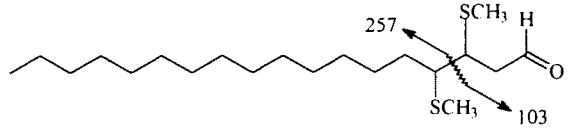
E- or Z-11-octadecenal DMDS adduct



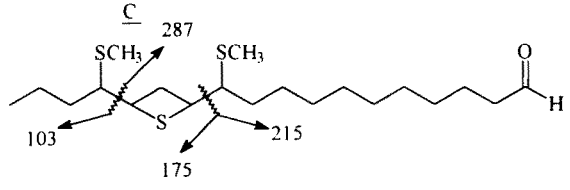
E- or Z-6-octadecenal DMDS adducts



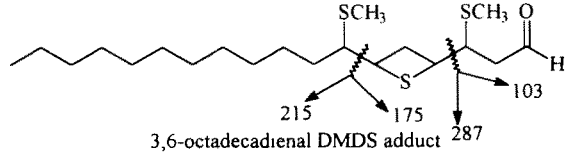
E- or Z14-octadecenal DMDS adduct



E- or Z-3-octadecenal DMDS adduct



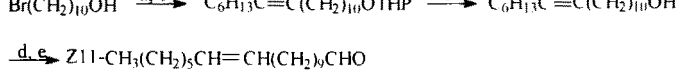
11,14-octadecadienal DMDS adduct



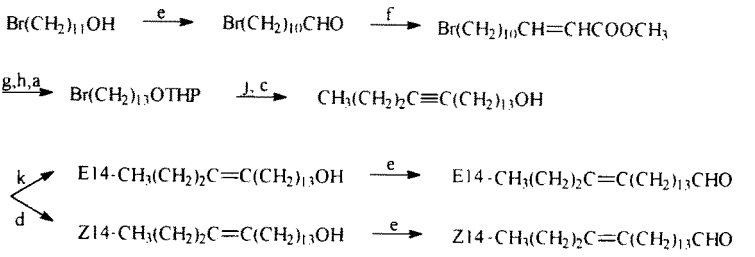
3,6-octadecadienal DMDS adduct

FIG. 2. Fragmentation patterns expected from DMDS adducts of candidate aldehydes in the gland extracts.

(A) Preparation of (Z)-11-octadecenal



(B) Preparation of (E)- and (Z)-14-octadecenal



(C) Preparation of (E,E)- and (Z,E)-11,14-octadecadienal

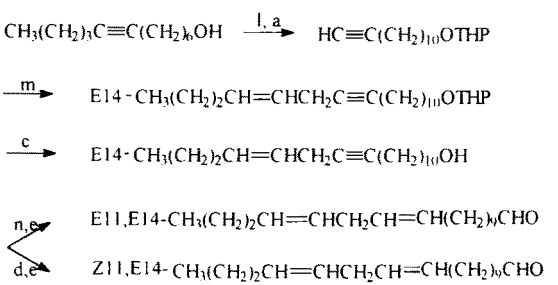


FIG. 3. Preparation of (*Z*)-11-octadecenal, (*E*)-14-octadecenal, (*Z*)-14-octadecenal, (*E,E*)-11,14-octadecadienal, and (*Z,E*)-11,14-octadecadienal. Reagents: (a) dihydropyrane/*p*-TSA (*p*-toluenesulfonic acid); (b) 1-octyne/*n*-BuLi (Schwarz and Waters, 1972); (c) MeOH/*p*-TSA; (d) H_2/Pd/$BaSO_4$; (e) PDC (pyridinium dichromate); (f) Wittig reaction, $Ph_3P{=}CHCO_2CH_3/CH_2Cl_2$; (g) H_2/Pd; (h) DIBAL (diisobutylaluminum hydride) (Yoon and Gyoung, 1985); (j) pentyne/*n*-BuLi (Schwarz and Waters, 1972); (k) $LiAlH_4$/diglyme (diethyleneglycol dimethyl ether) (Rossi and Carpita, 1977); (l) potassium 3-aminopropylamide (KAPA) (Brown and Yamashita, 1976); (m) (*E*)-2-bromohexene/*n*-BuLi (Schwarz and Waters, 1972); (n) Na/NH_3 (Schwarz and Waters, 1972). *Abbreviation:* THP, 2-tetrahydropyranyl.

the adduct showed a molecular weight of 392 and fragments at *m/z* 217 and 103 (Table 3). These results indicated that the alcohol was 11,14-octadecadienol, since 3,6-octadecadienol would give major fragments at *m/z* 215 and 105. Thus, it was concluded that component D in the gland extract was 11,14-octadecadienal. The four geometric isomers of 11,14-octadecadienal, i.e., (*E*,*E*)-, (*Z*,*E*)-, (*Z*,*Z*)-, and (*Z*,*E*)-11,14-octadecadienal, were synthesized and their retention times on GC were compared with that of the component D (Table 2). Cochromatography of (*E*,*E*)-11,14-octadecadienal with the natural product showed agreement on different columns. Thus, component D in the sex pheromone gland extract was identified as (*E*,*E*)-11,14-octadecadienal.

Pheromone Titer Determination and PBAN Stimulation Tests. Recently, Raina et al. (1989) reported that pheromone biosynthesis activating neuropeptide (PBAN) regulates pheromone production in some female insects. We found that the amount of the four components was zero if the adult female moth of the tea cluster caterpillar was decapitated and saline was injected into the abdomen (Table 4). In contrast, if PBAN was injected instead of saline into the neck-ligated and decapitated moth, the four components we have identified were found in the gland extract, and the percentage of *E*11,*E*14-18:Ald in the gland extract was as high as 75.5%. This indicated that all four compounds were probably related to pheromone production. The fact that the percentage content of *E*11,*E*14-18:Ald in the gland extract of the PBAN treated females was more than that of normal virgin females (55.1%) suggests that *E*11,*E*14-18:Ald is the sex pheromone of *Andraca bipunctata*.

EAG Analysis. The EAG analysis of octadecanal, (*E*)-11-octadecenal, (*E*)-14-octadecenal, and (*E*,*E*)-11,14-octadecadienal of different dosage showed that the antennae of the male strongly responded to (*E*,*E*)-11,14-octadecadienal and only slightly to the other three components (Figure 4). The EAG response of each component was measured only at doses of 200 and 300 ng since the response induced by 200 or 300 ng of synthetic chemicals was comparable to

TABLE 4. EFFECT OF PBAN ON PERCENTAGE OF FOUR COMPONENTS IN FEMALE PHEROMONE GLAND EXTRACT OF *A. bipunctata* WALKER

	18:Ald[a]	*E*11-18:Ald[a]	*E*14-18:Ald[a]	*E*11,*E*14-18:Ald[a]
Normal female (8)[b]	18.5 ± 4.3a	6.9 ± 1.2a	19.4 ± 2.4a	55.1 ± 4.9b
PBAN injected female (8)[b]	9.9 ± 2.3b	3.9 ± 1.1b	10.5 ± 1.3b	75.5 ± 3.3a
Ligated female (11)[b]	0	0	0	0

[a]Means followed by a different roman letter in the same column are significantly different ($P = 0.05$).

[b]Number of replicates indicated in parentheses.

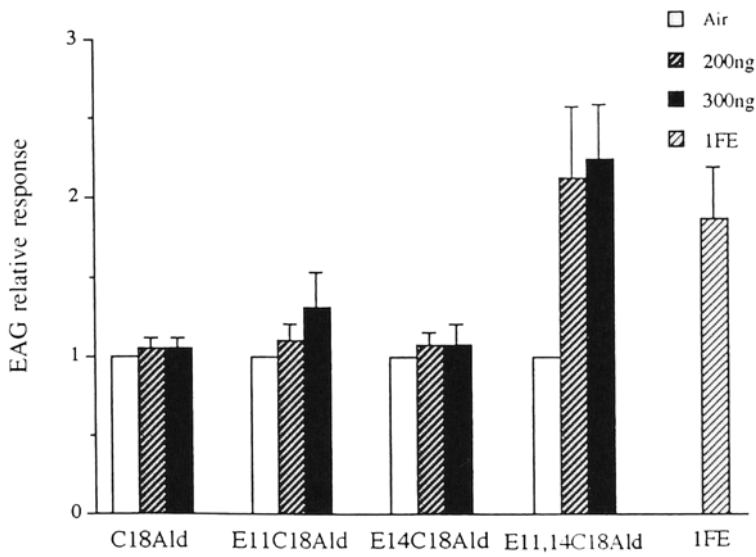


FIG. 4. EAG response of male *Andraca bipunctata* to 18:Ald, *E*11-18:Ald, *E*14-18:Ald, *E*11,*E*14-18:Ald, and 1 FE. The vertical bar indicates the standard error of the mean.

TABLE 5. NUMBER OF MALE *A. bipunctata* ATTRACTED TO SYNTHETIC COMPOUNDS, 18:Ald/*E*11-18:Ald/*E*14-18:Ald/*E*11, *E*14-18:Ald (20:8:31:41) AND VIRGIN FEMALE EXTRACT DURING NOVEMBER 6-16, 1994 AT TAIWAN TEA EXPERIMENT STATION, PUESHIN[a]

Date	18:Ald/*E*11-18:Ald/*E*14-18:Ald/*E*11,*E*14-18:Ald (20:8:31:41)		2 virgin female equivalent	Control
	0.1 mg	0.5 mg		
1994 Nov. 6	0	0	0	0
7	3	2	0	0
8	1	2	0	0
9	0	0	0	0
10	0	0	0	0
14	1	4	0	0
15	0	0	0	0
16	0	0	0	0
Total	5	8	0	0

[a]The lure dispenser was a plastic tube.

that of one female equivalent. The EAG results provided support for (*E*,*E*)-11,14-octadecadienal as the sex pheromone of the insect.

Field Trapping of Released Males. The results of field trapping of released males with mixtures of the synthetic compounds in a ratio of 20:8:31:41 and virgin-female extract are shown in Table 5. A total of 120 males were released and only 13 were caught. The reason for the poor recapture of males in this experiment was probably due to the low winter temperature when the trapping took place.

Field Trapping of Wild Males. The results of field trapping of wild males with each of the synthetic compounds and mixtures of the synthetic compounds are shown in Tables 6 and 7. The results in Table 6 showed that component D (*E*11,*E*14–18:Ald) alone, two-component treatments (A + D, B + D, C + D), and mixtures of four synthetic compounds (mixed in the same ratio as in the virgin female extract) caught more males than treatments without component D. The numbers of moths caught by the different treatments were not significantly different as long as component D was present. Table 7 shows the comparison of the number of males caught by three-component and four-component treatments. Although the adult season was almost over in May, and only a few male moths were caught in this test, the results also showed no difference between the three-component treatments and four-component treatments. The fact that the number of males captured by *E*11,*E*14–18:Ald alone and by mixtures of two, three, or four components were not significantly different from each other support EAG data and PBAN results, and we conclude that *E*11,*E*14–18:Ald is the attractive component of the sex pheromone gland of *Andraca bipunctata*. Although a single pheromonal component is rare in insect literature, it is not unprecedented. For example, female gypsy moths use only (7*R*,8*S*)-*cis*-7,8-epoxy-12-methyloctadecane to attract males. To see if there is any synergetic effect of the other components found in the gland extract, more extensive field tests will be conducted in the near future.

From our results of PBAN stimulation, EAG analysis, and field trapping tests, we tentatively conclude that the sex pheromone of *Andraca bipunctata* is *E*11,*E*14–18:Ald. If the additional three components in the gland extract do not have any function in male attraction, the reason they are present may be that they are precursors in the biosynthesis of (*E*,*E*)-11,14-octadecadienal. The moth may synthesize *E*11,*E*14–18:Ald from 18:Ald via *E*11–18:Ald or via *E*14–18:Ald in the pheromone gland. The Δ11 desaturase enzyme identified from some Lepidoptera may produce *E*11–18:Ald from the saturated 18:Ald (Roelofs and Wolf, 1988). A Δ14 desaturase is also reported to be involved in the biosynthesis of pheromone in the Asian corn borer (Zhao et al. 1990). Thus the sex pheromone *E*11,*E*14–18:Ald reported here could be produced by catalytic activities of the Δ11 and Δ14 desaturases in its biosynthetic pathway as reported in other insects.

TABLE 6. NUMBER OF MALE *Andraca bipunctata* ATTRACTED TO SYNTHETIC SEX PHEROMONE 18:Ald (A), *E*11-18:Ald (B), *E*14-18:Ald (C), *E*11,*E*14-18:Ald (D) DURING APRIL 1995 AT NANKANG TEA PLANTATION NEAR ACADEMIA SINICA[a]

	Treatment																										
	A			B			C			D			A+D			B+D			C+D			A+B+C+D			Control		
Date	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
1995 Apr. 5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	3	0	1	1	3	1	0	3	5	0	0	0
8	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	1	2	1	1	4	1	4	9	0	0	0
15	0	0	0	0	0	0	0	0	0	0	1	2	0	2	3	1	3	4	0	1	3	5	1	2	0	0	0
20	0	0	0	0	0	0	0	0	0	1	4	3	2	3	1	2	1	4	3	2	4	2	3	3	0	0	0
29	0	0	0	0	0	0	0	0	0	4	11	24	17	8	14	17	14	12	15	10	12	9	9	9	0	0	0
Total		0			0			0		6	17	29	21	14	20	24	19	23	20	17	24	17	20	28	0	0	0
Average[c]		0b			0b			0b			17.3a			18.3a			22a			20.3a			21.6a			0b	

[a]The lure dispenser was a plastic tube. Total amount of the chemicals was 0.1 mg. I, II, III indicate replicates.
[b]The ratio of the four chemicals was 20:8:31:41.
[c]Averages followed by the same letter are not significantly different by Duncan's multiple range test ($P = 0.05$).

TABLE 7. NUMBER OF MALE *Andraca bipunctata* ATTRACTED TO SYNTHETIC SEX PHEROMONE 18:Ald (A), *E*11-18:Ald (B), *E*14-18:Ald (C), *E*11,*E*14-18:Ald (D) DURING MAY 1995 AT NANKANG TEA PLANTATION NEAR ACADEMIA SINICA[a]

	A+B+D			A+C+D			B+C+D			A+B+C+D			Control		
Date	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
1995 May 8	0	2	2	2	0	1	3	1	2	1	2	2	0	0	0
15	1	0	0	1	1	0	1	1	2	0	1	1	0	0	0
25	3	1	5	2	1	0	0	1	4	2	4	5	0	0	0
30	0	1	0	0	1	2	2	0	1	1	3	0	0	0	0
June 5	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Total	4	4	7	5	3	3	6	3	9	4	10	9	0	0	0
Average[c]		5.0a			3.6ab			5.3a			7.6a			0c	

[a]The lure dispenser was a plastic tube. Total amount of the chemicals was 0.1 mg. I, II, III are replicates.
[b]The ratio of the four chemicals was 20:8:31:41.
[c]Averages followed by the same letter are not significantly different by Duncan's multiple range test ($P = 0.05$).

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SEMIOCHEMICALS VIA EPOXIDE INVERSION

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Abstract—A sequence of reactions is presented for inverting the configurations of both epoxide carbons in 1,2-disubstituted epoxides. As examples, (+)-disparlure was converted to its enantiomer, (−)-disparlure, and *exo-endo* conversion of a cyclohexene oxide was demonstrated.

Key Words—Enantiomer, chirality, pheromone, disparlure.

INTRODUCTION

(+)-Disparlure **1** [(7*R*,8*S*)-2-methyl-7,8-epoxyoctadecane] (Figure 1), the sex pheromone of the gypsy moth, *Lymantria dispar* L., (Bierl et al., 1970) is a chiral biomolecule whose performance in trapping experiments has been found to be very sensitive to the presence of even small amounts of the (−) enantiomer (Plimmer et al., 1977). We recently developed an analytical procedure for determining the chirality of **1** that involved conversion, with two inversions of configuration, to *N*-(α-methylbenzyl)aziridines (Oliver and Waters, 1995). This procedure involved epoxide opening with α-methylbenzylamine (stereoselective with inversion, but not regioselective), mesylation of the OH, and intramolecular displacement of the mesylate (stereoselective with inversion). It occurred to us that a similar sequence, but using an appropriate oxygen nucleophile, ought to be applicable to an epoxide–epoxide enantiomeric conversion that would complement the formal *cis–trans* inversions of disparlure developed in our labora-

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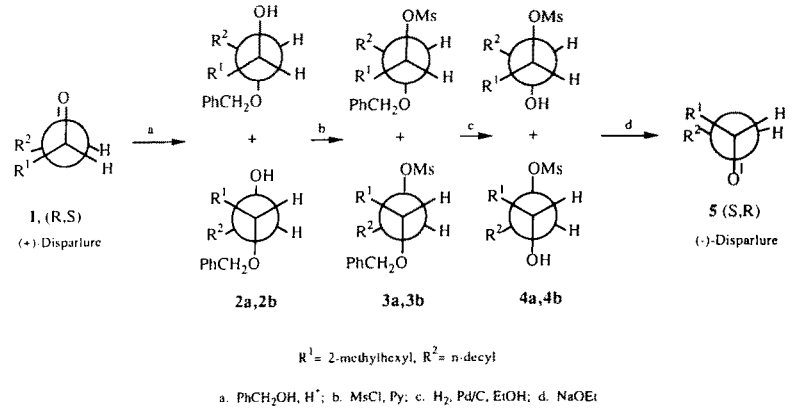


FIG. 1. Conversion of (+)-disparlure to (−)-disparlure.

tories a number of years ago (Sonnet and Oliver, 1976a,b). Here we report the application of epoxide inversion to the synthesis of (−)-disparlure and an unrelated epoxide of semiochemical interest.

METHODS AND MATERIALS

Gas chromatography was performed on Shimadzu 9A and 14A instruments, equipped with 15-m DB-5 and 60-m SPB 608 capillary columns, respectively. The former was generally used for following the progress of reactions and flash chromatography, whereas the latter, operated isothermally at 240°C, was employed for disparlure chirality analyses (Oliver and Waters, 1995) and also for attempted separations of positional isomers derived from epoxide opening of disparlure (e.g., **2a, 2b** and **3a, 3b**). ^{1}H NMR spectra were obtained with a General Electric QE-300 spectrometer on deuteriochloroform or deuteriobenzene solutions. Mass spectra were recorded on a Finnigan model 4510 GC-MS. (Mention of a proprietary product or company does not imply endorsement by the U.S. Department of Agriculture.)

Inversion of (+)-Disparlure to (−)-Disparlure. A mixture of (+)-disparlure (170 mg), benzyl alcohol (466 mg), and 2 Nafion beads (Aldrich, ca. 10 mg) was allowed to stand at room temperature for four days, then was thoroughly partitioned between pentane and water. Flash chromatography (2.5% then 5% EtOAc in cyclohexane) provided separation from rearrangement products and gave 155 mg (66%) of a mixture of regioisomeric benzyloxy alcohols **2a** and **2b.** [No GC separation of the ketonic rearrangement products was realized;

chemical ionization mass spectrometry confirmed molecular weight 282 (isomeric to **1**); the electron impact spectrum contained the four expected acyl ions for 2-methyloctadecan-7-one and 2-methyloctadecan-8-one (*m/z* 127, 141, 169, 183) but the predicted McClafferty rearrangement ions (*m/z* 142, 156, 184, 198) although visible, were too weak to be very diagnostic: m/z(%), 183 (13), 169 (16), 127 (10), 123 (10), 109 (23), 96 (14), 95 (19), 85 (18), 83 (15), 82 (100), 71 (57), 67 (11), 59 (13), 58 (39), 57 (73), 55 (34). Its infrared spectrum (neat, 1712 cm^{-1}) indicated ketone(s), as did the lack of a deshielded signal in the ^1H NMR spectrum indicative of an aldehyde.] The entire sample of **2a** and **2b** in pyridine (2 ml) was treated with methanesulfonyl chloride (100 μl) in a nitrogen atmosphere, and the solution was allowed to stand overnight at room temperature. It was then partitioned between cold, dilute HCl and pentane, and the pentane was rinsed with water and with aqueous sodium carbonate. Removal of solvent provided 203 mg of **3a** + **3b** as a clear oil that was not purified (TLC yielded a single spot, R_f = 0.43, 5% EtOAc/toluene), but was hydrogenated (1 atm, 10% Pd on charcoal) in absolute ethanol—see below. No gas chromatographic resolution was achieved between regioisomers **2a, 2b,** or **3a, 3b,** and detailed characterization was not attempted. Mass and ^1H NMR spectra were recorded on mixtures of the isomers: Mass spectrum **2a** + **2b** (GS introduction; mol wt 390 confirmed by chemical ionization with both methane and ammonia reagent gases) electron ionization m/z (%) 390 (0.03, M^+), 127 (6), 92 (14), 91 (100), 83 (5), 69 (10), 57 (6), 55 (7). Mass spectrum of **3a** + **3b** (probe introduction): m/z (%), 468 (0.1, M^+), 127 (6), 111 (8), 107 (10), 97 (9), 92 (7), 91 (100), 83 (10), 69 (17), 57 (10), 55 (10).

Following hydrogenation of **3a, 3b,** filtration and evaporation of solvent gave 111 mg of **4a** + **4b** as a glassy residue. Addition of methanol (2 ml) followed by 0.25 ml of 2.9 M sodium methoxide in methanol resulted in the almost immediate precipitation of a white solid. After 1.75 hr the solvent was concentrated, and the residue was partitioned between water and pentane. Flash chromatography of the pentane-soluble product (hexane followed by 2.5% and finally 5% ethyl acetate in hexanes) gave 67 mg of (−)-disparlure **5** indistinguishable from the starting material except for its chirality (Oliver and Waters, 1995—see text). Mass spectrum of **5** (e. i., m/z (%)]: 282, M^+ (0.2), 183 (8), 111 (17), 110 (17), 109 (10), 97 (42), 95 (17), 85 (9), 84 (11), 83 (27), 82 (58), 71 (17), 70 (22), 71 (17), 70 (22), 69 (100), 67 (25), 57 (38), 56 (39), 55 (64).

Opening of Ethyl-trans-4-Methyl-7-oxabicyclo[4.1.0]heptane-3-cis-Carboxylate **6.** A mixture of **6** (racemic, 7.36 g), benzyl alcohol (30 ml), and Nafion (0.5 g) was allowed to stand at room temperature for 12 days (this reaction could also be conducted at elevated temperatures if desired). The product was partitioned between pentane and water, and then, after evaporation of

the pentane, between cyclohexane and water. Alternatively, the bulk of the excess benzyl alcohol could be removed by distillation *in vacuo* prior to partitioning. Removal of solvent gave 9.87 g of a clear oil that contained **8** plus its positional isomer and very little benzyl alcohol. Mass spectrum of **8:** m/z (%), 168 (8), 155 (8), 137 (6), 111 (13), 109 (10), 94 (12), 92 (15), 91 (100), 85 (8), 83 (6), 81 (20), 71 (14), 65 (10), 55 (9).

Benzyloxymesylate **9.** The preceding sample, dissolved in a mixture of pyridine (15 ml) and dichloromethane (30 ml), was stirred and cooled in a nitrogen atmosphere while a solution of methanesulfonyl chloride (3.5 ml) in dichloromethane (10 ml) was added dropwise. After 2.5 hr at 0°C the ice bath was removed, and after an additional hour 0.5 ml of methanesulfonyl choride was added slowly. After stirring at room temperature one more hour, ice was added and the mixture was partitioned between water and ether–hexane (1 : 1). The solution was rinsed with water, twice with 2 N HCl, again with water, and finally with 1 M Na_2CO_3. The solution was dried and concentrated, and the residue (12 g) was passed through a short silica gel column with 10% followed by 20% ethyl acetate in hexanes. Evaporation of solvent gave 10.46 g (84%) of **9** plus its regioisomeric benzyloxy mesylate. A sample of the major isomer **9** was isolated by flash chromatography (ca. 20% ethyl acetate in hexanes). Mass spectrum of **9:** m/z (%), 370 (1, M^+), 168 (17), 122 (6), 95 (6), 94 (38), 92 (8), 91 (100), 81 (7), 65 (6). ^1H NMR of **9** ($CDCl_3$) 0.94 (d, J = 6.6, 6-Me), 1.24 (t, J = 7.2, $C\underline{H}_3CH_2$), 1.73 (m, H-$5_{a,b}$), 1.96 (m, H-2), ca. 2.05 (m, H-6), 2.40 (dt, J = 5.4 and 10.2 H-1), 2.98 (s, $C\underline{H}_3SO_3$), 3.79 (app. d, H-3), 4.15 (q, J = 7.2, $CH_3C\underline{H}_2$), 4.55 (d, J = 11.7, $PhC\underline{H}_2$), 4.62 (d, J = 11.7, $PhC\underline{H}_2$), 4.84 (app. d, J = 3.0, H-4), 7.34 (phenyl).

Hydrogenolysis. A solution of 1.47 g of **9** in absolute ethanol (20 ml) plus 0.17 g 10% Pd on C was hydrogenated at one atmosphere. No reaction occurred, and eventually the mixture was filtered and the catalyst was replaced with ca. 0.2 g of PtO_2. Three drops of conc. HCl were added, but no hydrogenation occurred until the solution was again filtered and the catalyst replaced with 0.25 g of fresh PtO_2. After two more hours, GC indicated the disappearance of **9** and the appearance of two products, **10** and **11,** with the **10** predominating by about 2 : 1. The mixture was filtered through Celite, and the filtrate was treated with 2 ml of 3 N NaOEt in ethanol. After 0.5 hr the mixture was partitioned between aqueous ammonium chloride and 8 : 2 hexane–ethyl acetate. Removal of solvent gave 0.63 g of an oil that was flash chromatographed (10%, 20%, and 30% EtOAc in hexanes) to give 277 mg of epoxide **7** and 215 mg of cyclohexanemethanol derivative **11.** The epoxide **7** was identical to **7** produced along with **6** upon epoxidation of the corresponding olefin. Epoxide **7:** m/z (%) 184 (0.7, M^+), 139 (32), 138 (10), 114 (14), 112 (12), 111 (34), 110 (100), 101 (21), 95 (24), 93 (53), 91 (10), 86 (10), 85 (14), 84 (34), 83 (24), 82 (15),

81 (41), 79 (13), 77 (11), 73 (18), 72 (19), 71 (10), 70 (25), 69 (25), 68 (13), 67 (33), 56 (13), 55 (71), 53 (15). ^{1}H NMR ($CDCl_3$) 0.84 (d, J = 6, 6-Me), 1.25 (t, J = 7.2, $C\underline{H}_3CH_2O$), 1.52 (m, H-$5_{a,b}$), 1.66 (m, H-6), 2.01 (m, H-$2_{a,b}$), 2.13 (m, H-$5_{b,a}$), 2.23 (m, H-1), 2.27 (m, H-$2_{a,b}$), 3.14 (app. t, H-4), 3.22 (br s, H-3), 4.13 (dq, J = 1.2 and 6.9, $CH_3C\underline{H}_2O$). Compound **11** was identified by its mass and NMR spectra: m/z (%) 331 (6), 207 (10), 185 (45), 183 (30), 155 9260, 139 (12), 138 (8), 137 (22), 111 (55), 109 (11), 97 (62), 96 (13), 95 (16), 94 (17), 93 (25), 81 (22), 79 (17), 65 (11), 63 (13), 55 (100), ^{1}H NMR ($CDCl_3$) 0.92 (d, J = 6.6, 6-Me), 1.27 (t, J = 6.9, $C\underline{H}_3CH_2$), 1.65 (m, H-$5_{a,b}$), 1.85 (m, H-$5_{a,b}$), 1.92 (m, H-2), 2.32 (dt, J = 4.8 and 10.2, H-1), 3.04 (s, CH_3SO_2), 3.24 (dd, J = 6.6 and 9.0) and 3.31 (dd, J = 6.6 and 8.7), $C\underline{H}_2$-cyclohexyl, 3.62 (br d, H-3), 4.15 (q, J = 7.2, $CH_3C\underline{H}_2O$) 4.81 (br, H-4).

*Confirmation of Cyclohexanemethanol Derivative **11**.* A solution of **6** (166 mg) and pyridinium *p*-toluenesulfonate (23 mg) in cyclohexanemethanol (1 ml) was heated as 110°C under nitrogen ca. 20 hr. Part of the cyclohexanemethanol was removed in vacuo, and the residue was thoroughly partitioned between pentane and water. To about 90% of the crude product was added benzene (2 ml), pyridine (0.5 ml), and methanesulfonyl chloride (0.5 ml). After 3 hr at room temperature the mixture was partitioned between water and ether–hexane (1 : 1). The organic phase was rinsed with dilute HCl, with water, and with aqueous Na_2CO_3, and then dried and concentrated. Flash chromatography (20–25% EtOAc–hexane gave a total of 90 mg of **11**, part of which was crystallized from hexane, mp 60–61. This material was identical to **11** isolated from the above reaction.

RESULTS AND DISCUSSION

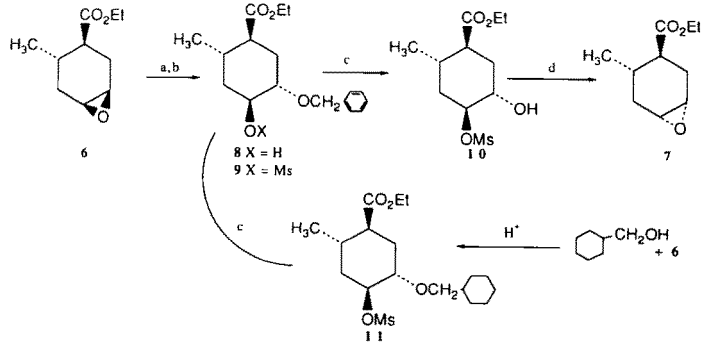
We had noted (Oliver and Waters, 1995) that disparlure (**1**) was very resistant to ring opening under nonacidic conditions, but that anhydrous neutral alumina (Posner and Rogers, 1977) satisfactorily catalyzed ring opening with α-methylbenzylamine at 145°C. Under similar conditions (sealed tube), alumina catalyzed epoxide opening with benzyl alcohol. Rearrangement (the rearrangement of **1** is assumed to proceed more or less equally to two regioisomeric ketones, but like other regioisomers in the disparlure series, no gas chromatographic resolution was achieved—see Methods and Materials) competed with opening, but the products were readily separated by flash chromatography and **2a, 2b** were isolated in 34% yield. Other catalysts were also investigated: a trace of sulfuric acid quickly catalyzed rapid opening of **1** in benzyl alcohol at room temperature, but considerable self-condensation of benzyl alcohol also

occurred [product(s) not investigated] Nafion-catalyzed opening (Olah et al., 1981) with benzyl alcohol as solvent was slow (four to seven days at room temperature for complete reaction), but epoxide rearrangement was minimized ($\leq 15\%$), and **2a, 2b** were obtained in 66% yield after chromatography. Pyridinium toluenesulfonate seemed quite similar to Nafion as a catalyst, although a systematic comparison was not made.

Mesylation of regioisomers **2a, 2b** proceeded without incident, as did hydrogenolysis (EtOH, Pd on C, 1 atm) of the benzyloxymesylates **3a, 3b.** Treatment of the resulting hydroxymesylates **4a, 4b** with sodium ethoxide resulted in rapid conversion to expoxide **5** (7*S*,8*R*), indistinguishable from **1** by gas chromatography or mass spectrometry. The yield of **5,** after flash chromatography, from **3a, 3b** was 81% (Figure 1).

For successful conversion of **1** to **5,** both the epoxide opening and closing reactions have to occur with inversion. If one of the two were to occur with retention, the result would be *trans*-disparlure (7*R*,8*R*) or (7*S*,8*S*). *trans*-Disparlure is separable from **1** and **5** by GC or even column chromatography, and, in fact, ca. 0.6% of the *trans* epoxide was detectable (by GC) in at least one conversion of **1** to **5.** The starting material for this sequence had been 97.3% **1** and 2.7% **5,** and the product analyzed for 2.2% **1** and 97.8% **5.** It is uncertain whether this 0.5% difference is outside of experimental error, but in any event, the stereoefficiency was very high.

Another example of epoxide inversion involved **6** and **7** (Figure 2). These had long ago been found to be attractive to Mediterranean fruit flies (Valega and Beroza, 1967), but only a mixture of *exo* and *endo* epoxides is available upon epoxidation of *trans*-6-methyl-3-cyclohexenecarboxylic acid or its esters. In contrast, the *exo*-epoxide **6** is readily available via an iodolactone (see, for



a. $PhCH_2OH$, H^+; b. CH_3SO_2Cl, Py; c. H_2, Pt, EtOH; d. NaOEt

FIG. 2. The *exo-endo* epoxide inversion.

example, Ogliaruso and Wolfe, 1993), but comparison of the attractiveness of pure **6** and the mixture of **6** and **7** suggested that the activity lay with the latter (R.T. Cunningham and J.W. Avery, unpublished results). Epoxide **6** (in this case racemic) was less subject to rearrangement than disparlure, and ring opening could conveniently be carried out at 100–110°C with either pyridinium toluenesulfonate or Nafion in benzyl alcohol. In this case the opening was somewhat regioselective, and after treatment of the crude product with methanesulfonyl chloride, mesylate **9** could be isolated as a solid. Benzyloxymesylate **9** was more resistant to hydrogenation than **3a, 3b.** No reaction was observed with palladium on carbon in ethanol (1 atm), whereas addition of a trace of HCl and replacement of the catalyst with platinum oxide permitted a very slow reduction to occur (in some cases requiring two or more days to reach completion at one atmosphere). The major product was evidently the expected alcohol **10,** since treatment of the ethanol solution with sodium ethoxide rapidly gave the desired epoxide **7.** No isomers of **7** were detected, although a minor product was observed from the conversion of **9** to **7.** After isolation by flash chromatography, it was identified by mass spectrometry and NMR spectroscopy as the cyclohexylmethyl ether **11,** i.e., the product of aromatic ring reduction instead of hydrogenolysis. Platinum-catalyzed ring saturation of benzyl ethers, although generally unanticipated, is not without precedent (see Rylander, 1979). The structure of **11** was confirmed by opening **6** with cyclohexanemethanol instead of with benzyl alcohol, then treating the ether–alcohol with methanesulfonyl chloride.

In summary, a straightforward procedure for the inversion of certain 1,2-disubstituted epoxides is reported. Although asymmetric epoxidation procedures can often be applied to either enantiomer, particularly of acyclic epoxides, many cyclic epoxides, naturally occurring epoxides, or epoxides prepared by microbial- or enzyme-mediated sequences, may be more available as one enantiomer than the other (Besse et al., 1994), and the current method may prove useful in such cases. We have not investigated inversions of trisubstituted epoxides, anticipating that epoxide rearrangements as well as difficulties with tertiary mesylates could present competing pathways. Unsaturated substrates subject to catalytic hydrogenation would present another limitation of the sequence described, but alternative oxygen nucleophiles convertible to OH by other means, such as *p*-methoxyphenol (Corey and Link, 1992), or masked OH nucleophiles (Fleming, 1988) might also find application. Ironically, an initial objective of the project was to invert the configuration of (+)-diethylepoxyfumarate. We were unable to effect ring opening of that epoxide with benzyl alcohol under a variety of acidic or alkaline conditions; only very slow (over several weeks) transesterification occurred under the conditions described above for the opening of **1** or **7,** and the corresponding dibenzyl ester (examined separately) failed to react at all.

Acknowledgments—We are grateful to Dr. P.E. Sonnet for his suggestion of inverting the stereochemistry of the epoxyfumarates and for supplying both the diethyl and dibenzyl esters. We thank Dr. James Avery for a sample of epoxide **6** and for a mixture of **6** and **7.**

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MULTIVARIATE DISCRIMINATION OF HOST USE BY DWARF MISTLETOE *Arceuthobium vaginatum* subsp. *cryptopodum*: INTER- AND INTRASPECIFIC COMPARISONS

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Abstract—The parasitic dwarf mistletoe *Arceuthobium vaginatum* attacks *Pinus ponderosa* as its primary host and *P. contorta* as an occasional host. Within ponderosa pine stands there is also differential parasitism among individual trees. We compared biochemical features of phloem and xylem oleoresin between infected individuals of the two pine species ($N = 15$ for each species) and also between infected ($N = 30$) and nearby uninfected ($N = 30$) ponderosa pine conspecifics. There were significant differences in chemical features, both at the interspecific (*P. ponderosa* vs. *P. contorta*) and intraspecific (*P. ponderosa*) levels. Discriminant function analysis based on chemical features of phloem correctly classified all trees used in the analysis as either *P. ponderosa* or *P. contorta*, and 95% of all ponderosa pine trees as either parasitized or nonparasitized. Monoterpene composition of oleoresin was distinct between species, and differences between parasitized and nonparasitized *P. ponderosa* were also significant. Many of the observed chemical differences are probably constitutive, although levels of nonstructural carbohydrates and α-pinene may change in response to dwarf mistletoe infection. Biochemical differences at the intraspecific level were distinct from interspecific differences. Patterns of differential attack can have genetic consequences upon both the parasite and its hosts, and, in the process, may contribute to the evolution of host races of the parasite and to the evolution of host resistance within ponderosa pine.

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Key Words—Dwarf mistletoe, *Arceuthobium vaginatum*, ponderosa pine, *Pinus ponderosa*, lodgepole pine, *Pinus contorta*, parasitic plants, monoterpenes, differential selection, host resistance, host chemistry.

INTRODUCTION

The differential utilization of hosts by parasites is often affected by chemical features of the host phenotype. When parasites are animals, they may move about the landscape sampling different potential hosts and make active choices about which individual hosts to attack. Host choice can be based in part on nutritive value or chemical repellency of individual hosts, or some combination of both (e.g., Denno and McClure, 1983; Fritz and Simms, 1992; Snyder, 1992). When parasites are plants (or microbes) and are dispersed passively by seeds or spores, patterns of attack do not involve such active choices. Nonetheless, patterns of attack by passively dispersed parasites may also be mediated by specific features of host chemistry, since parasites may be differentially successful (e.g., germination, survival, reproduction) on hosts with different phenotypes (Scharpf et al., 1992; Linhart et al., 1994). Variability of chemical features of hosts is often poorly known and needs to be documented in detail to gain a better understanding of host–parasite interactions.

To investigate the potential role of host chemistry in these interactions, we examined patterns of attack by the dwarf mistletoe *Arceuthobium vaginatum* subsp. *cryptopodum* (Viscaceae) and chemical characteristics of its known and potential hosts. The chemical features we examined are known to be associated with patterns of use by a variety of herbivores and parasites of these trees (Snyder, 1992, 1993; Snyder and Linhart, 1994; Linhart et al., 1994). *Arceuthobium vaginatum* resides in the Rocky Mountains and the southwestern United States. Its primary host species is ponderosa pine (*Pinus ponderosa* subsp. *scopulorum*), but it utilizes lodgepole pine (*P. contorta*) as an occasional host (Hawksworth and Wiens, 1972). Within forest stands, the distribution of dwarf mistletoes is usually patchy (Scharpf and Parmeter, 1978; Hawksworth and Johnson, 1989), but even in stands where it is very dense, certain trees can be heavily infected, while nearby trees often are free from parasitism (Linhart, 1989; Linhart et al., 1995). These observations suggest that there may be differences in biochemical features of host and nonhost trees. Such differences may be important to dwarf mistletoe establishment and survival after seeds germinate and send "sinkers" into the phloem and xylem of potential hosts, since dwarf mistletoes obtain all of their water and elemental nutrients and the majority of their carbohydrate resources from host trees. Once established, dwarf mistletoe individuals can live up to several years on their hosts. They show differential mortality (Scharpf et al., 1992) when introduced experimentally onto host trees

known to be resistant or susceptible, suggesting that biochemical characteristics of hosts may affect dwarf mistletoe survivorship.

In order to determine whether host biochemistry is associated with patterns of attack and establishment by dwarf mistletoe, we compared the biochemical features of phloem and of xylem oleoresin between infected individuals of ponderosa and lodgepole pines. We also compared these same chemical features between infected ponderosa pines and nearby ponderosa pine individuals free from infection. We asked the following specific questions:

1. At the interspecific level, what biochemical features of phloem and xylem oleoresin differentiate infected trees of the primary host (*P. ponderosa*) and occasional host (*P. contorta*) of *A. vaginatum*?

2. At the intraspecific level, is attack by *A. vaginatum* associated with particular biochemical features of individual *P. ponderosa* trees?

3. To what extent do interspecific and intraspecific differences in host characteristics correspond? That is, in ponderosa pine, do parasitized trees differ from nonparasitized trees in the same ways that ponderosa pine, the primary host, differs from lodgepole pine, the occasional host?

METHODS AND MATERIALS

Locations and Sample Sizes. We used two study sites in the southern Rocky Mountains, one for intraspecific comparisons, another for interspecific comparisons. Trees for the intraspecific comparisons ($N = 15$ pairs) occupied a stand of ca. 5 ha of ponderosa pine at 2182 m elevation on Bald Mountain, 6.1 km west of Boulder, Colorado. For these comparisons, we designated pairs of trees (i.e., 30 heavily parasitized and 30 trees with no visible symptoms of dwarf mistletoe infection) that were matched for size (within 10% of each other in height and diameter), that were near each other, and subject to similar soil type, aspect, and solar exposure. The trees designated for sampling in the interspecific comparison were intermixed in a stand of ca. 3 ha at 2591 m elevation in the Roosevelt National Forest 2 km north of Nederland, Colorado, and included both infected and noninfected trees of both species. The stand consisted of a relatively dense mixture of both host species. Neither site was characterized by significant heterogeneity of soils or parent materials.

Collection and Processing. For all trees, two live branches 1–3 cm in diameter that showed no visible symptoms of dwarf mistletoe infection were collected from separate locations within the tree crown using an extendable rope-pull tree pruner. Branches were brought to the laboratory and stored at 5°C until processing (<48 hr). Phloem was peeled from twigs with a drawknife and scalpel, air-dried, and pulverized to a fine powder in a steel, motorized grinder. All samples were analyzed for percentage of total nonstructural carbohydrates

using a phenol–sulfuric acid colorimetric method that determines percent total nonstructural carbohydrate expressed as percent glucose equivalents (Hodge and Hofreiter, 1962). Concentrations of aluminum, boron, calcium, cadmium, copper, iron, manganese, magnesium, nitrogen, phosphorus, potassium, silicon, sodium, titanium, and zinc were determined using an inductively coupled plasma spectrometer as described in Snyder (1992).

Oleoresin flow rates were measured for intraspecific comparisons only. Rates were determined by tapping the xylem of trees according to methods modified slightly from those of Smith (1977) as detailed by Snyder (1992). Within each site, all trees were tapped within a 3-hr period on the same day because flow rates can vary with ambient temperature (Smith, 1977). Xylem oleoresin collected from individual trees was analyzed subsequently by gas–liquid chromatography for percentage composition of nine monoterpenes found in measurable quantity (α-pinene, β-phellandrene, β-pinene, camphene, 3-carene, limonene, myrcene, γ-terpinene, and terpinolene). Handling and analysis of oleoresin samples were performed according to methods modified slightly from Smith (1977) as detailed in Snyder (1992).

RESULTS

Chemical characteristics of phloem differed significantly between ponderosa and lodgepole pines (Table 1). Eleven of 15 elements showed significant differences, and 13 of 15 elements were found at higher concentrations in phloem of lodgepole pine than in phloem of ponderosa pine. Monoterpene characteristics of oleoresin also differed markedly, with ponderosa pine containing variable levels of nine monoterpenes that were found in measurable amounts (α-pinene, β-phellandrene, β-pinene, camphene, 3-carene, limonene, myrcene, γ-terpinene, and terpinolene), and lodgepole pine characterized by measurable quantities of only β-phellandrene (Table 1). Discriminant function analysis based on chemical characteristics of phloem correctly classified 100% of trees as either *P. ponderosa* or *P. contorta*; levels of boron and zinc contributed most to the discriminant function.

At the intraspecific level, there were significant differences in phloem characteristics between ponderosa pines infected with dwarf mistletoe and those free from infection (Table 2). Seven of 15 elements showed differences significant at the 0.05 level. Discriminant fucntion analysis based on chemical features of phloem correctly classified 95% of the trees (57 of 60) used in the analysis as parasitized or nonparasitized. Relative concentrations of boron, magnesium, and manganese (Table 2) contributed most to the discriminant function. At the intraspecific level, discriminant function analysis based on xylem oleoresin characteristics (monoterpene composition and flow rate) correctly classified 62% of

TABLE 1. PHLOEM AND XYLEM CHARACTERISTICS OF PONDEROSA PINE AND LODGEPOLE PINE AT NEDERLAND SITE

Variable	Ponderosa pine (Mean ± SE)	Lodgepole pine (Mean ± SE)	P
Phloem features			
Aluminum[a]	399.00 ± 24.59	612.00 ± 60.94	<0.01
Boron	17.56 ± 0.56	21.28 ± 0.41	<0.01
Calcium	8773.00 ± 1062.56	16198.00 ± 2264.04	<0.01
Cadmium	0.53 ± 0.06	2.08 ± 0.25	<0.02
Copper	3.58 ± 0.38	4.42 ± 2.08	NS
Mercury	0.31 ± 0.96	0.05 ± 0.69	<0.01
Iron	39.96 ± 9.88	35.81 ± 4.87	NS
Manganese	99.10 ± 10.59	238.62 ± 17.53	<0.01
Magnesium	1398.00 ± 42.78	1821.00 ± 67.05	<0.01
Nitrogen	3812.00 ± 264.00	4059.00 ± 177.58	NS
Phosphorus	647.20 ± 49.48	795.53 ± 42.32	<0.01
Potassium	4025.00 ± 348.39	3555.00 ± 307.64	NS
Silicon	30.80 ± 2.27	43.33 ± 3.82	<0.01
Sodium	47.66 ± 3.28	55.46 ± 7.90	NS
Titanium	0.56 ± 0.05	1.04 ± 0.09	<0.01
Zinc	41.98 ± 2.72	82.58 ± 3.30	<0.01
TNC[b]	17.00 ± 0.28	16.00 ± 0.34	NS
Water[b]	57.00 ± 0.67	53.00 ± 0.57	<0.01
Xylem oleoresin monoterpenes[c]			
α-Pinene	9.80 ± 2.0	0	
β-Pinene	24.10 ± 2.2	0	
3-Carene	52.60 ± 2.2	0	
β-Phellandrene	1.00 ± 0.1	100	
Limonene	1.80 ± 0.5	0	
γ-Terpinene	0.20 ± 0.0002	0	
Terpinolene	2.90 ± 0.2	0	
Camphene	0.06 ± 0.01	0	
Myrcene	6.50 ± 0.70	0	

[a] Dry mass (μg/g) for all elements.
[b] Total nonstructural carbohydrates expressed as percentage dry mass; H_2O expressed as percentage
[c] Monoterpenes expressed as percentage composition

the trees used in the analysis as parasitized or nonparasitized. There were significant differences in percentage composition of both α-pinene and camphene and in flow rate between parasitized and nonparasitized *P. ponderosa* (Table 2).

Patterns of chemical differences between *P. ponderosa* and *P. contorta* were not consistent with differences between parasitized and nonparasitized *P.*

TABLE 2. PHLOEM AND XYLEM OLEORESIN CHARACTERISTICS OF PONDEROSA PINE PARASITIZED BY *Arceuthobium* OR FREE OF ATTACK

Variable	Attacked (Mean ± SE)	Not attacked (Mean ± SE)	*P*
Phloem features			
Aluminum[a]	76.97 ± 9.08	120.99 ± 11.41	<0.001
Boron	20.35 ± 0.63	16.17 ± 0.47	<0.0001
Calcium	6239.93 ± 382.15	6536.83 ± 576.48	NS
Cadmium	0.32 ± 0.02	0.33 ± 0.33	NS
Copper	1.99 ± 0.18	4.33 ± 0.98	<0.02
Mercury	0.13 ± 0.07	0.03 ± 0.07	NS
Iron	23.93 ± 2.64	24.83 ± 1.96	NS
Manganese	67.80 ± 3.66	97.76 ± 5.99	<0.0001
Magnesium	1350.10 ± 30.33	1176.4 ± 28.29	<0.0001
Nitrogen	3570.00 ± 200.00	3390.00 ± 200.00	NS
Phosphorus	653.7 ± 33.60	628.8 ± 17.91	NS
Potassium	4124.07 ± 21.19	3946.33 ± 112.38	NS
Silicon	58.87 ± 4.44	48.30 ± 3.25	<0.06
Sodium	66.03 ± 4.65	65.53 ± 6.30	NS
Titanium	0.86 ± 0.06	0.66 ± 0.06	<0.02
Zinc	43.97 ± 1.69	45.60 ± 1.50	NS
TNC[b]	18.71 ± 0.24	16.97 ± 0.54	<0.005
Xylem oleoresin characteristics[c]			
α-Pinene	10.04 ± 2.42	5.33 ± 0.25	<0.045
β-Pinene	21.48 ± 1.68	21.05 ± 1.82	NS
3-Carene	54.09 ± 2.35	58.37 ± 2.21	NS
β-Phellandrene	0.79 ± 0.13	0.99 ± 0.14	NS
Limonene	3.98 ± 0.95	4.53 ± 0.84	NS
γ-Terpinene	0	0	
Terpinolene	2.85 ± 0.23	3.14 ± 0.18	NS
Camphene	0.03 ± 0.01	0.09 ± 0.02	<0.0001
Myrcene	6.67 ± 0.73	6.48 ± 0.75	NS
Flow rate[d]	1.53 ± 0.37	3.02 ± 0.47	<0.03

[a] Dry mass (μg/g) for all elements.
[b] Total nonstructural carbohydrates expressed as percentage dry mass.
[c] Monoterpenes expressed as percentage composition.
[d] Flow rate expressed as milliliters per 24 hr.

ponderosa. Approximately half the elements in phloem show the same trend at the intra- and interspecific levels (Tables 1 and 2). β-Phellandrene, the only monoterpene found in measurable quantity in xylem oleoresin of *P. contorta*, was not found in significantly different percentage compositions in parasitized and nonparasitized *P. ponderosa*.

DISCUSSION

When dwarf mistletoes disperse their seeds in multiple directions, the propagules can land on many potential hosts. The random component of dispersal may be overcome by differential mortality on various hosts, as evidenced by variable densities of infection even on adjacent trees. Our results indicate that adjacent trees, whether they are the same or different species, often have very different biochemical features and that some of these features can be associated with the presence or absence of dwarf mistletoe colonies. Discriminant function analysis based on phloem chemistry correctly classified 100% of the trees used in the interspecific analysis as either *P. ponderosa* (primary host) or *P. contorta* (occasional host). These findings might be expected when comparing biochemical features of different species. However, our intraspecific comparisons of parasitized and nonparasitized trees showed that differences in biochemical features separated them with nearly the same level of discrimination as in the interspecific comparisons: discriminant function analysis based solely on phloem chemistry correctly classified 57 of the 60 trees (95%) as either parasitized or nonparasitized. This high level of discrimination suggests that host biochemistry may be at least partly responsible for the patchy distribution of the dwarf mistletoe *A. vaginatum* within populations of its primary host, *P. ponderosa*.

In order for host biochemistry to be an important determinant of dwarf mistletoe establishment, the differences we have documented between host and nonhost trees must exist prior to dwarf mistletoe attack; that is, they must be constitutive. An alternative is that these differences have somehow been induced by infection, perhaps representing defensive responses or other parasite-induced chemical/physiological interactions. We believe that most of the differences are, in fact, constitutive and reflect a genetically based biochemical heterogeneity. There is very strong evidence from ponderosa pine (Smith, 1977), other pine species (Squillace, 1971), and many other plants (e.g., Vernet et al., 1986) that there is a well-defined genetic basis for oleoresin monoterpene composition and flow rate; indeed the production of specific monoterpenes is controlled by single loci in the species referred to. There is also strong evidence that elemental accumulation in various plant tissues, including phloem, has a hereditary basis; this is also true both for conifers (e.g., MacLean and El Kassaby, 1986) and other plants (e.g., Roy, 1982; Clark, 1983). The demonstrated hereditary bases of phenotypic biochemical variability in ponderosa pine, even in the absence of dwarf mistletoe involvement (e.g., Smith, 1977; Snyder, 1992; Snyder and Linhart, 1994; Linhart et al., 1995), indicate that these stands consist of highly variable trees, i.e., comprise a highly heterogeneous substrate upon which dwarf mistletoes can potentially germinate and survive.

An alternative explanation to the chemical differences between host and

nonhost trees is that they are induced by dwarf mistletoe attack. This explanation may be relevant for at least three specific features of these trees: levels of carbohydrates and of α-pinene and the oleoresin flow rate. Dwarf mistletoes are known to secrete hormones that can lead to the accumulation of carbohydrates near the site of infection (Livingston et al., 1984). Therefore the higher levels of nonstructural carbohydrates we detected in parasitized trees may reflect this phenomenon, although we were careful to collect phloem well away from visibly infected sites and only from branches that showed no visible signs of infection. Levels of α-pinene and other monoterpenes may sometimes increase following attacks by insects such as *Dendroctonus* bark beetles (Lieutier et al., 1989) and in response to mechanical wounding (Marpeau et al., 1989). Oleoresin flow rate is known to have a strong hereditary component, but there is also evidence that trees stressed either by drought or parasitic attacks can have reduced rates of flow (Scharpf and Parmeter, 1978; Smith, 1977).

A potential complication in comparing characteristics of parasitized and nonparasitized trees is that apparently uninfected trees may have dwarf mistletoe endophytic growth with no external evidence of infection. However, in such trees infection is clearly in remission or at very low levels and has little impact upon tree health. Further, we attempted to minimize any potential complications from undetected endophytic growth by comparing trees that had no visible sign of infection with carefully matched trees that supported large numbers (20 to several hundred) of dwarf mistletoe individuals.

In summary, we are aware of the possibility that induction and other ecological factors such as soil heterogeneity may provide a partial explanation for some of the observed differences. However, we believe most of the differences we see are constitutive for the following reasons: (1) in pines, including ponderosa pine, monoterpene induction is responsible for only a small fraction of the documented heterogeneity (Squillace, 1971; Smith, 1977); (2) the trees we sampled grew within small areas, with relatively homogeneous soil and exposure characteristics; and (3) it appears unlikely that pines undergo simultaneously significant selective accumulation (e.g., B, Mg, Tn) and elimination (e.g., Al, Cu, Mn) of individual elements in response to parasite attacks. We are presently engaged in further tests of the possible importance of induction. The most definitive experiments involve experimental infection of trees with the parasite, and testing of tree biochemistry prior to and at intervals following infection.

Differences in biochemical characteristics of the primary (*P. ponderosa*) and secondary (*P. contorta*) host species do not correspond to differences between host and nonhost ponderosa pine. The two host species are biosystematically quite different, belonging to different subsections of the genus (Critchfield and Little, 1966). These differences are associated with differences in biochemical composition: lodgepole pine phloem contains markedly higher

levels of many elements than ponderosa pines growing intermixed with them, suggesting some fundamental differences in uptake/processing of elements, while nonstructural carbohydrate levels are remarkably similar. In addition, the almost uniform oleoresin monoterpene profiles of lodgepole pines contrast sharply with the tree-to-tree heterogeneity of ponderosa pine. Parasitized ponderosa pines, compared with nonparasitized conspecifics, had higher concentrations of some elements and monoterpenes and lower concentrations of others. It is hardly surprising, then, that intra- and interspecific comparisons do not match. These results point out that the heterogeneity of the potential environment for *A. vaginatum* is very great, and contributes to the complex selection pressures facing these organisms.

The patterns of differential establishment and host utilization we describe may have several important genetic consequences. At the interspecific level, the marked chemical differences in phloem and xylem oleoresin between ponderosa and lodgepole pines provide the opportunity for genetic differentiation between dwarf mistletoe subpopulations that parasitize these two host species. We have evidence for such differentiation at the Nederland site and at another site in Colorado where *A. vaginatum* parasitizes both lodgepole and ponderosa pine (Linhart et al., 1994). There is evidence that host race formation can occur in other dwarf mistletoe species although it may not necessarily occur under conditions of sympatry (e.g., Nickrent and Stell, 1990).

At the intraspecific level, the dwarf mistletoe *A. vaginatum* can create significant selective pressures upon ponderosa pine populations. This parasite is by far the most damaging disease agent of ponderosa pine in the southwestern United States. It routinely reduces the growth and reproductive output of its host trees by 50% or more compared with uninfected trees and often kills its hosts in the long run (Hawksworth and Wiens, 1972; Scharpf and Parmeter, 1978; Linhart and Snyder, in preparation). This reduction in host fitness, coupled with evidence that there is a strong hereditary basis for elemental accumulation (MacLean and El Kassaby, 1986) and oleoresin composition (Squillace, 1971; Smith, 1977) in various conifers, suggests a possible mechanism for the evolution of host resistance to this parasite in ponderosa pine. Such resistance to *Arceuthobium* has indeed been reported in both ponderosa and the related Jeffrey pine (*P. jeffreyi*) (e.g., Roth, 1974; Scharpf et al., 1992).

In conclusion, (1) biochemical features of both phloem and xylem oleoresin differ significantly between the primary and occasional host of *A. vaginatum*. This may provide the opportunity for genetic differentiation between dwarf mistletoe subpopulations that parasitize different host species. (2) Significant biochemical differences between parasitized and non-parasitized ponderosa pines may be partly responsible for the patchy distribution of attack by *A. vaginatum* within populations of its primary host. (3) The lack of congruence between these

intra- and interspecific chemical differences underscores the complex nature of selection pressures facing *A. vaginatum*.

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VOLATILE INFOCHEMICALS USED IN HOST AND HOST HABITAT LOCATION BY *Cotesia flavipes* CAMERON AND *Cotesia sesamiae* (CAMERON) (HYMENOPTERA: BRACONIDAE), LARVAL PARASITOIDS OF STEMBORERS ON GRAMINAE

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Abstract—Female *Cotesia flavipes* Cameron and *Cotesia sesamiae* (Cameron) were attracted to odors in a Y-tube olfactometer from uninfested maize (*Zea mays* L.), sorghum [*Sorghum bicolor* (L.)], and napier grass (*Pennisetum purpureum* Schumach). In dual choice tests, *Cotesia flavipes* showed a preference for maize over sorghum, while maize and napier grass were equally attractive. In contrast, *Cotesia sesamiae* preferred volatiles from sorghum and napier grass over those from maize. The two parasitoids were significantly more attracted to maize infested with the stemborers, *Chilo partellus* (Swinhoe), *Chilo orichalcociliellus* Strand, *Sesamia calamistis* Hampson, and *Busseola fusca* (Fuller), than uninfested maize. In dual choice tests, *Cotesia flavipes* and *Cotesia sesamiae* were unable to discriminate between odors from plants infested by the different species of stemborers.

Key Words—Host location, host habitat location, *Cotesia flavipes*, *Cotesia sesamiae*, Hymenoptera, Braconidae, cereals, parasitoid, stemborers, Lepidoptera, Pyralidae, Noctuidae, Y-tube olfactometer.

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INTRODUCTION

Classical biological control has been used as a strategy for suppressing populations of introduced lepidopteran pests in the tropics. The success of classical biological agents has been attributed in part to the high searching efficiency of the natural enemy for its host (Waage, 1990). The ability of a natural enemy to locate a suitable host in a complex environment is critical to its successful establishment in a new environment (Nordlund et al., 1988). The host selection process is divided into three steps: host habitat location, host location, and host acceptance (Vinson, 1976). Allelochemicals are known to play an important role in this process. Chemicals emitted by plants influence the searching behavior of parasitoids (Nordlund et al., 1988; Lewis and Martin, 1990; Vet and Dicke, 1992; Dicke, 1994; Godfray, 1994). In addition, females of many insect parasitoids may rely on host and host-related chemicals (e.g., from frass) in their search for hosts (Vinson, 1976; Weseloh, 1981; Arthur, 1981; Vet and Dicke, 1992). However, host-produced chemicals often play a much less important role in long-distance searching by parasitoids than do plant-produced chemicals (Turlings et al., 1991a,b; Vet and Dicket, 1992; Steinberg et al., 1993; Geervliet et al., 1994; Potting et al., 1995a).

Chilo partellus (Swinhoe) (Pyralidae) is one of the major lepidopterous stemborer pests of cereal crops in Kenya (Overholt et al., 1994a). This pest was introduced into Africa sometime in the early 1930s (Tams, 1932). Indigenous natural enemies, including the larval parasitoid, *Cotesia sesamiae* (Cameron), are not able to maintain the pest population density at a level acceptable to farmers (Oloo, 1989; Overholt et al., 1994a). *Cotesia flavipes* Cameron, which has been used successfully in classical biological control of gramineous stemborers in other parts of the world (Overholt et al., 1994b), was introduced into Kenya from Pakistan in an attempt to reduce the severity of the stemborer problem.

Chilo partellus is found in cultivated gramineous plants together with indigenous stemborer species, which include *Chilo orichalcociliellus* Strand (Pyralidae), *Busseola fusca* (Fuller), and *Sesamia calamistis* Hampson (Noctuidae). The same complex of stemborer species is also found on wild grasses (Mathez, 1972; Seshu Reddy, 1989; Harris, 1990). Little is known about the foraging behavior of *Cotesia flavipes* and *Cotesia sesamiae* in the system comprising the stemborers and plants mentioned above. It was reported that *Cotesia flavipes* cannot discriminate between maize infested with *Chilo partellus* and maize infested with *B. fusca* (Potting et al., 1993). However, *Cotesia flavipes* showed a preference for maize stems with feeding *Chilo partellus* larvae over artificially damaged maize, larvae alone, host frass, or uninfested maize (Potting et al. 1995a). It is not known whether this parasitoid is attracted to the different

gramineous plants (which are the food of its hosts, e.g., maize, sorghum, napier grass) occurring in the area of release, the coast of Kenya. It is also not known whether *Cotesia flavipes* has a preference for any of these host plants either uninfested or infested by larvae of different stemborer species (*Chilo orichalcociliellus, Chilo partellus, S. calamistis*) occurring in that area. No information is available on the host selection process of the closely related indigenous parasitoid, *Cotesia sesamiae*. The extent of overlap in the niches of the two *Cotesia* species also is not known. The aim of this study was, therefore, to investigate the response of *Cotesia flavipes* and *Cotesia sesamiae* to volatiles from uninfested maize, sorghum and napier grass and the response of both parasitoids to volatiles from different stemborer–plant complexes that are likely to be found in the area of release.

METHODS AND MATERIALS

Parasitoids

A colony of *Cotesia flavipes* was started in 1991 from material collected from *Chilo partellus* at Rawalpindi, Pakistan, by the International Institute of Biological Control (IIBC). The *Cotesia sesamiae* colony was also started in 1991 with material collected from *Chilo partellus* in the coastal zone of Kenya. *Cotesia flavipes* and *Cotesia sesamiae* were reared on *Chilo partellus* and *S. calamistis* larvae, respectively, according to the method described by Overholt (1993). *Chilo partellus* and *S. calamistis* are among the respective natural hosts of *Cotesia flavipes* and *Cotesia sesamiae*. After parasitization, stemborers were maintained on artificial diet (Ochieng et al., 1985; Onyango and Ochieng'-Odero, 1994) at 25°C, 65–70% relative humidity, and 12L:12D photoperiod. Parasitoid cocoons were collected in glass vials and kept in a clean Perspex cage until emergence of adults. Adult parasitoids were provided a 20% honey–water solution as diet. One-day-old, mated, naive female parasitoids were used in all experiments.

Stemborers

Chilo partellus, S. calamistis, and *Chilo orichalcociliellus* were collected from maize fields near the Kenyan coast. A colony of *B. fusca* was initiated with larvae and pupae collected from maize in western Kenya. Larvae of *Chilo* spp. were reared on an artificial diet developed by Ochieng et al. (1985). The noctuids, *B. fusca* and *S. calamistis*, were reared on media described by Onyango and Ochieng'-Odero (1994). Prior to any experiment, stemborer larvae were removed from artificial diet and fed on fresh maize stems for 48 hr.

Plants

Maize (*Zea mays* L., hybrid 5-11) and sorghum [*Sorghum bicolor* (L.), Serena cultivar], were grown in 20-liter plastic buckets in a nursery at the International Centre of Insect Physiology and Ecology (ICIPE). Potted plants were kept under large field cages (2 × 2 × 2 m), which were covered with fine mesh (400 μm) netting to protect them from insect attack. Additional maize plants were grown in the field. Napier grass (*Pennisetum purpureum* Schumach.) was collected from farmers' fields near Nairobi.

Behavior Assays

The Y-tube olfactometer used in this study has been described by Steinberg et al. (1992). The odor sources were placed in two Perspex chambers (30 × 30 × 120 cm) sufficiently large to accommodate whole plants (2–3 months old) (Figure 1). One of the square ends of the chamber was left open. For the system

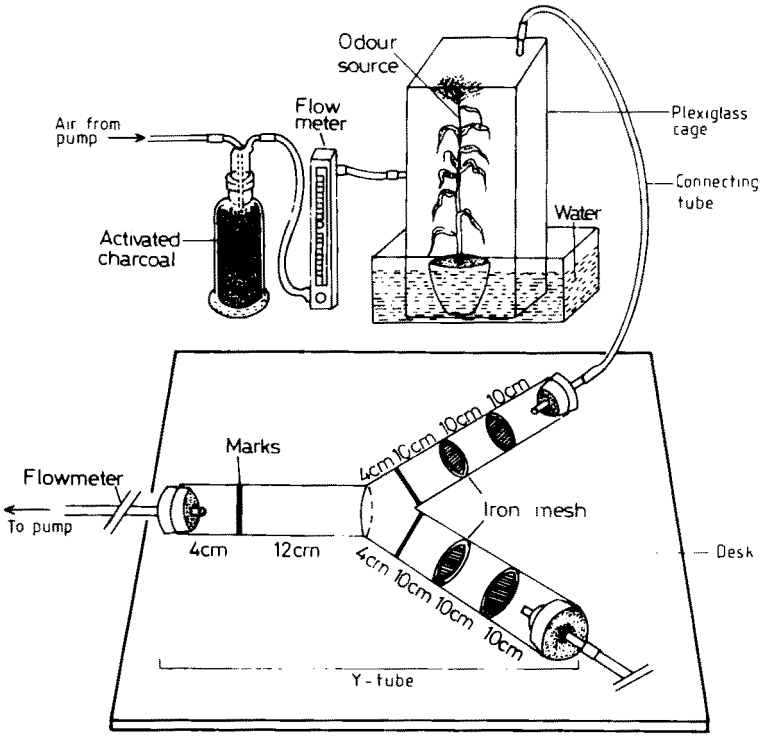

FIG. 1. A schematic diagram of the Y-tube olfactometer.

to be airtight, the open end of each box was placed over the test material, which stood in water held in a plastic basin. The open end was submerged 15 cm below the meniscus. The two chambers were connected to the arms of the Y-tube with Tygon tubing from the top of the chambers. An inlet, through which clean air entered the chamber, was drilled 30 cm from the bottom of the chamber on one side. One vacuum pump (Cole-Parmer Air-Cadet) drew and pushed air through the closed system. Air was pushed through an activated charcoal filter into the two chambers and drawn into the Y-shaped glass tubing of the olfactometer. The airflow was set at 2.5 liters/min for each arm. Parasitoids were released individually in the stem of the Y-tube and allowed 5 min to choose one of the arms. When a parasitoid remained more than 15 sec beyond the finishing line (4 cm past the intersection), it was recorded as a choice. The connections of the odor source chambers to the arms of the olfactometer were reversed after testing five insects to rule out the effect of asymmetrical bias in the olfactometer or its surroundings. A cream-white curtain was used to separate the experimental area from the surroundings. Tests were conducted at 23–26°C, 65–75% relative humidity, and light intensity of 350–450 lux. All tests were replicated at least three times with 20 parasitoids per replicate. Data were analyzed using the log likelihood ratio test (G test) for goodness of fit (Sokal and Rohif, 1981).

Response to Volatiles from Uninfested Plants. Uninfested maize, sorghum, and napier grass plants 8–10 weeks old were used in the experiments. All plant species were tested in single and dual choice experiments in the Y-tube olfactometer.

A first series of experiments was conducted to determine the attractiveness of odors from uninfested plants. Maize, napier grass, and sorghum were tested in single choice experiments. Individual parasitoids were given a choice between odor from potted maize and air drawn over a pot with soil only, odors from potted sorghum plant and air drawn over a pot with soil only, and finally odor from napier grass cut above the soil level and air drawn over an empty chamber.

A second series of tests was conducted to determine the parasitoid's preference for odors from different uninfested plant species. Plants were placed in chambers connected to both arms of the olfactometer. An approximately similar biomass of plants was used for each arm. Attractiveness of volatiles from potted maize was compared with attractiveness of volatiles from potted sorghum, and odors from maize plants from the field excised above soil level were compared with that of napier grass treated the same way. Field materials were checked for damage before each test and dissected after a test to establish it was uninfested.

Response to Volatiles from Plants Infested by Different Stemborer Species. In the Y-tube olfactometer, parasitoids were given a choice between odors from plants infested with different stemborer species [plant–herbivore complex (PHC)]

and uninfested plants. The following PHC combinations were tested against the control (uninfested plants): (1) maize plants infested with *Chilo partellus*, (2) maize plants infested with *Chilo orichalcociliellus*, and (3) maize plants infested with *S. calamistis*. Potted maize plants were infested with two fourth instars by boring two holes in the maize stem (1 cm deep) with a 4-mm cork borer and placing one larva in each hole. Larvae were allowed to feed overnight, and tests were conducted 18–20 hr after infestation. Holes were also made in control plants, but no larvae were introduced. Plants were transferred into the chambers connected to the Y-tube in the pot in which they were grown 30 min before observations were made (to allow time for volatiles to be released in the chamber). Only plants grown in pots were used in this test. In a few cases potted plants that were too tall to enter the test chamber were excised and tested. Plants in both arms were treated in the same manner (cut at the base or kept in pots).

A second series of tests was conducted to determine the parasitoids' preference between plants infested by different stemborer species. The following combinations were tested: (1) maize infested with *Chilo partellus* larvae versus maize infested into *Chilo orichalocociliellus* larvae, (2) maize infested with *Chilo partellus* larvae versus maize infested with *S. calamistis* larvae, and (3) maize infested with *Chilo partellus* larvae versus maize infested with *B. fusca* larvae [only *Cotesia sesamiae* was used for the test with *B. fusca; Cotesia flavipes'* response having already been studied by Potting et al. (1993)].

A third series of tests was conducted to determine the response of parasitoids depending on the number of stemborer larvae feeding of the plant. The following number/species combinations of stemborers per plant were compared: (1) two *Chilo partellus* versus 10 *S. calamistis*, (2) five *Chilo partellus* versus five *S. calamistis*, (3) 10 *Chilo partellus* versus two *S. calamistis*, and (4) two *B. fusca* versus ten *Chilo partellus* larvae.

RESULTS

Response to Volatiles from Uninfested Plants. Cotesia flavipes and *Cotesia sesamiae* were more attracted to odors from gramineous plants (maize, sorghum, napier grass), the habitat of their host, than to the control, clean air (Figure 2). *Cotesia flavipes* did not exhibit a preference between maize and napier grass (Figure 3). However, maize was preferred over sorghum by *Cotesia flavipes*, whereas *Cotesia sesamiae* preferred sorghum over maize (Figure 3). The preferences of the two parasitoids significantly differed (contingency table, $\chi^2 = 16.71$, $df = 1$; $p < 0.001$); *Cotesia sesamiae* also preferred napier grass over maize (Figure 3).

Response to Maize Plants Infested by Different Stemborer Species. Parasitoids of both species preferred odors from maize plants infested with two larvae

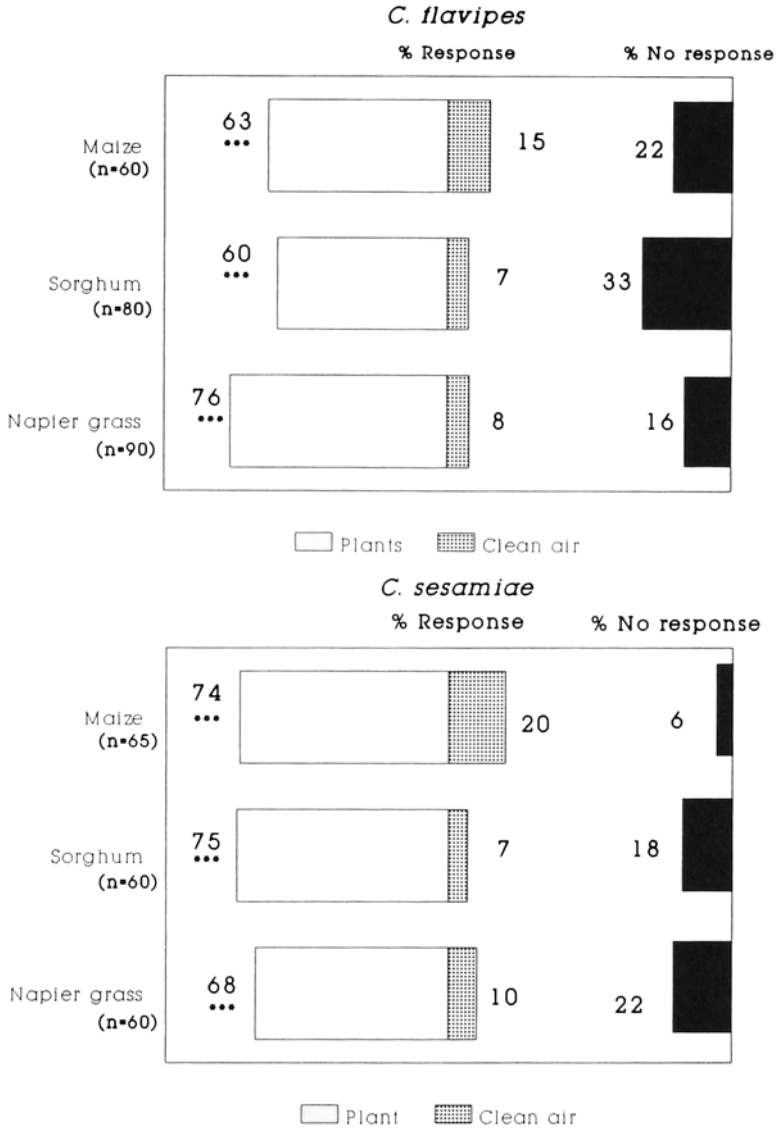

FIG. 2. Response of naive *Cotesia flavipes* and *Cotesia sesamiae* to odors from uninfested plants versus clean air in a Y-tube olfactometer. N = number of replicates. Numbers next to the bars indicate the percentages of parasitoids that made a choice for one of the two odor sources or did not make a choice at all. The three percentages add up to 100%. Asterisks indicate significant differences within the choice test: $*P < 0.05$, $**P < 0.01$; $***P < 0.001$, ns = not significant, G-test for goodness of fit.

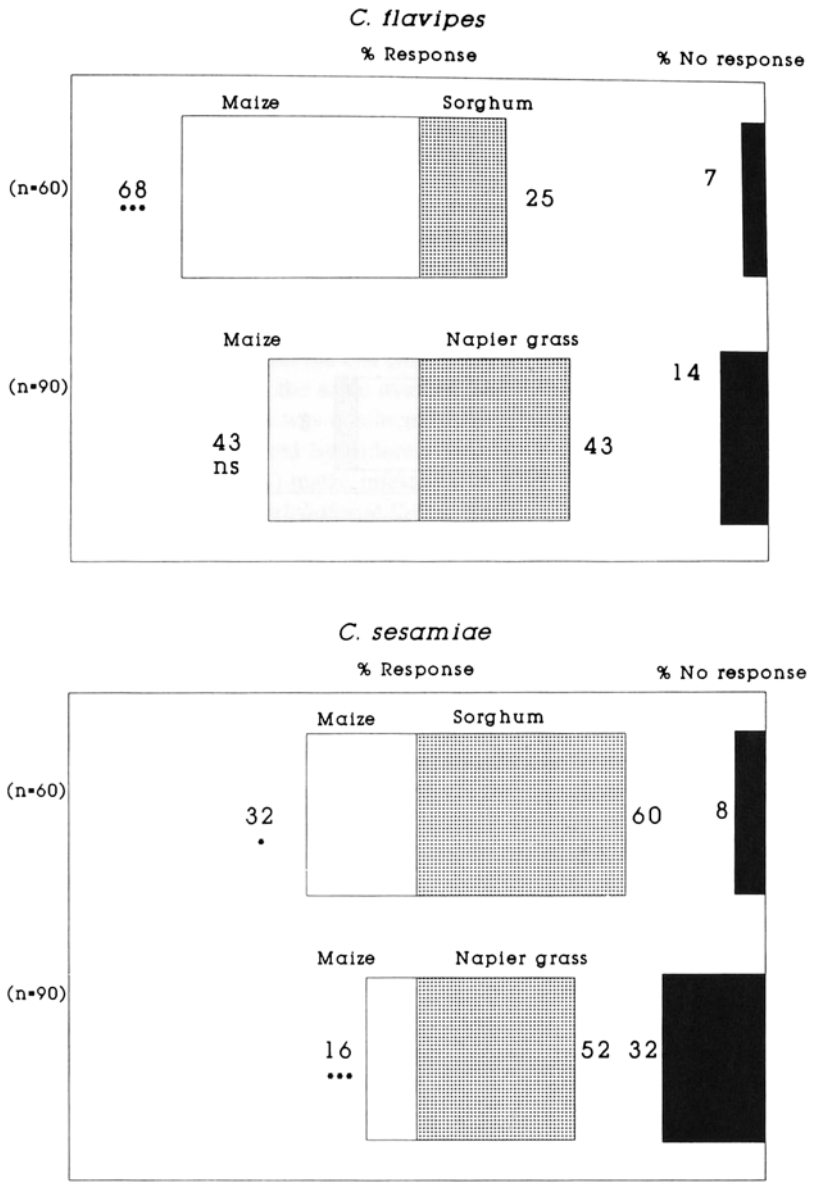

FIG. 3. Response of naive *Cotesia flavipes* and *Cotesia sesamiae* to odors from uninfested plants in two-choice tests in the Y-tube olfactometer. For other explanations see legend of Figure 1.

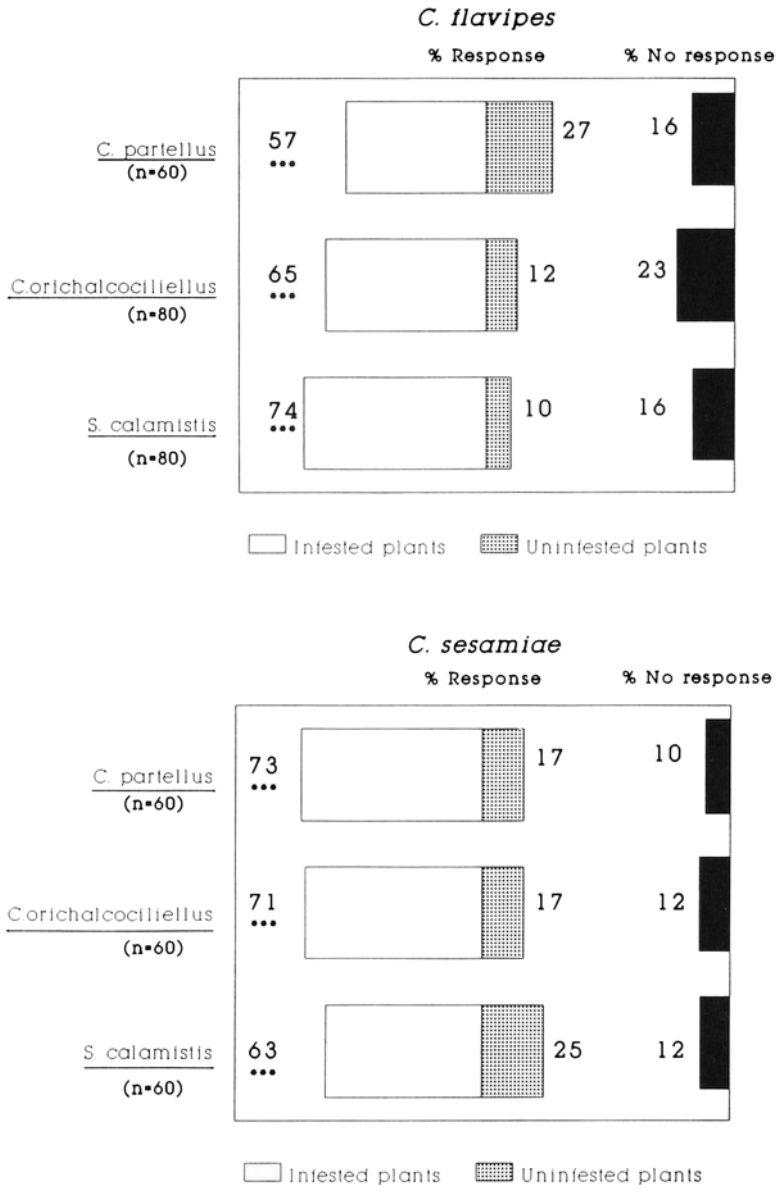

FIG. 4. Response of naive *Cotesia flavipes* and *Cotesia sesamiae* to odors from maize plants infested with different stemborer species versus uninfested maize plants in a Y-tube olfactometer. For other explanations see legend of Figure 1.

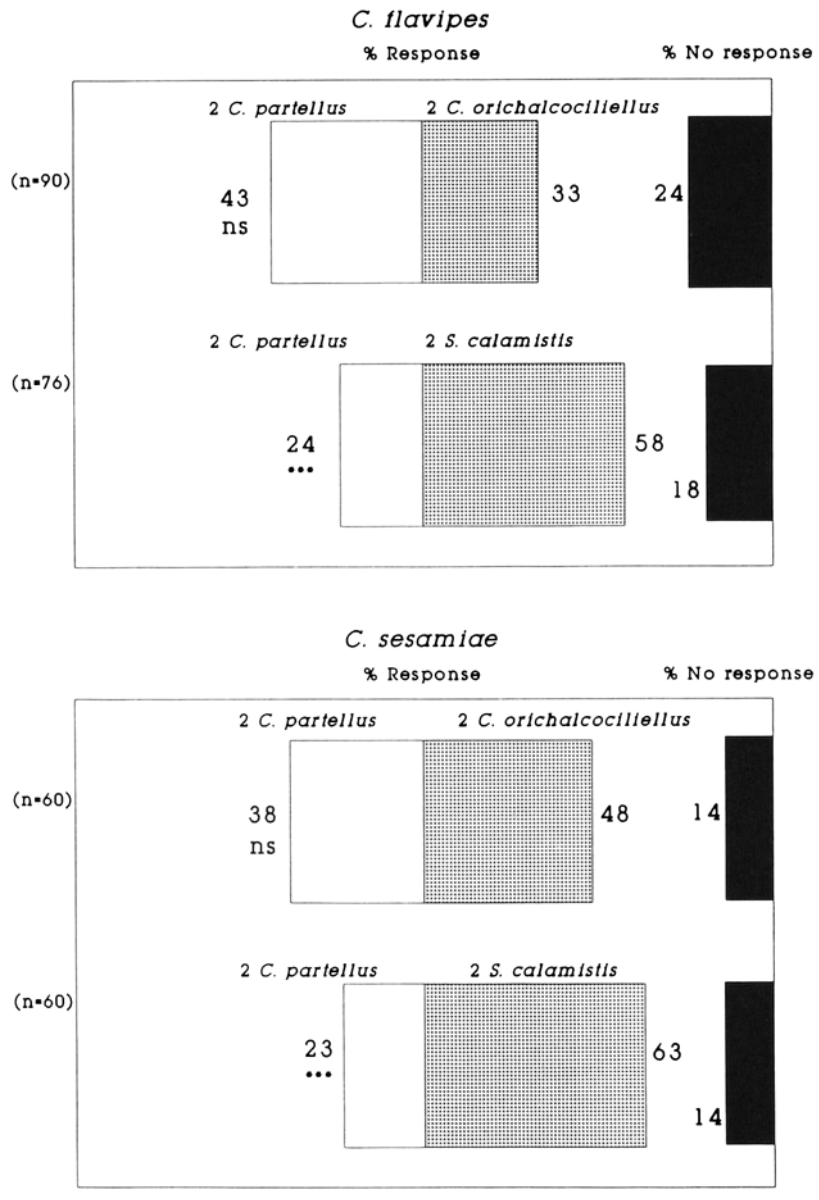

FIG. 5. Response of naive *Cotesia flavipes* and *Cotesia sesamiae* in the Y-tube olfactometer to odors from maize plants infested with two stemborer larvae per plant, in two-choice tests. For other explanations see legend of Figure 1.

of *Chilo partellus*, *S. calamistis*, or *Chilo orichalcociliellus*, per plant, over odors from uninfested maize plants (Figure 4).

When maize plants infested with two larvae of one of two species were offered in dual choice situations, both parasitoid species preferred odors of plants infested by *S. calamistis* over those infested with *Chilo partellus*. Neither parasitoid species discriminated between maize plants infested with two *Chilo partellus* larvae and maize plants infested with two *Chilo orichalcociliellus* larvae (Figure 5).

Several other combinations were tested with *Chilo partellus* and *S. calamistis* as herbivores. When maize plants infested by two larvae of one species were offered versus plants infested by 10 larvae of the other, *Cotesia flavipes* preferred odors from maize plants infested with 10 larvae in both cases. When odors from plants infested with five *Chilo partellus* larvae were offered versus odors from plants infested with five *S. calamistis* larvae, *Cotesia flavipes* females did not discriminate (Figure 6).

Cotesia sesamiae preferred odors from maize plants infested with two larvae of *B. fusca* over odors from uninfested maize. This parasitoid also preferred

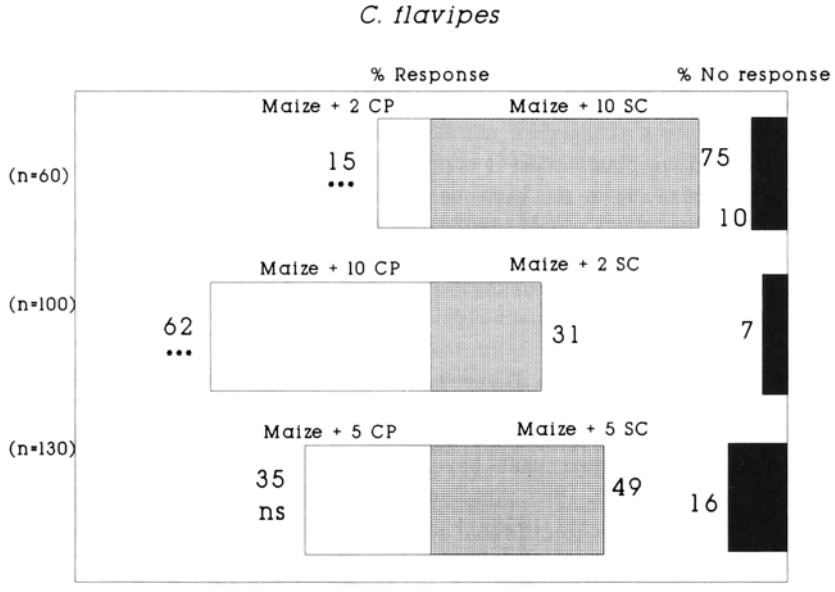

FIG. 6. Response of naive Cotesia flavipes in the Y-tube olfactometer to odors from maize plants infested with larvae of *Chilo partellus* (CP) or *Sesamia calamistis* (SC) at different densities. For othr explanations see legend of Figure 1.

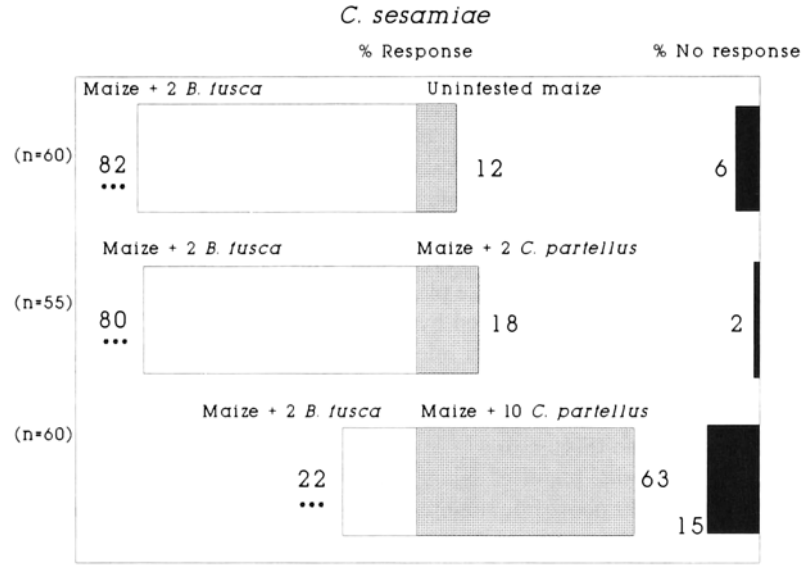

FIG. 7. Response by naive *Cotesia sesamiae* in the Y-tube olfactometer to odors from uninfested maize plants and maize plants infested with larvae of *B. fusca* and plants infested with larvae of *Chilo partellus* or *B. fusca* at different densities. For other explanations see legend of Figure 1.

odors from maize plants infested with *B. fusca* over odors from those infested with *Chilo partellus* at the same density (two larvae per plant) (Figure 7). However, when plants where infested with 10 *Chilo partellus* larvae and two *B. fusca* larvae, *Cotesia sesamiae* showed preference for odors emanating from plant infested with *Chilo partellus* (Figure 7).

DISCUSSION

Plant odors play an important role in communication in tritrophic systems. Volatiles from uninfested plants have been found to be long-range attractants for parasitoids (Vinson, 1985; Nordlund et al., 1988; Whitman, 1988; Williams et al., 1988; Lewis et al., 1990). For example, *Campoletis sonorensis* (Cameron) is attracted to uninfested food plants of its hosts (Elzen et al., 1983). *Macrocentrus grandii* Goidanich, a larval parasitoid of the European corn borer, *Ostrinia nubilalis* (Hübner), is also attracted to several plant species including its host's food, uninfested maize (Ding et al., 1989). In the present study, *Cotesia flavipes* and *Cotesia sesamiae* were strongly attracted to odors emanating

from several gramineous plants (maize, sorghum, and a wild host, napier grass), which are host plants of stemborers. In all single-choice tests, the gramineous plant was selected over the control, regardless of the plant species. *Cotesia flavipes* showed a preference for maize over sorghum in the dual choice experiments. This parasitoid was imported from Pakistan, where it was collected from *Chilo partellus* in maize fields. This preference may, therefore, be a reflection of a genetic adaptation to searching in maize. In contrast, the indigenous parasitoid *Cotesia sesamiae* was more attracted to sorghum odors, and this may have been due to a genetic adaptation as well: there has been a long history shared by *Cotesia sesamiae* and sorghum, which has its center of origin in Africa (Doggett, 1988). Except for a few records showing *Cotesia flavipes* attacking *Diacrisia obliqua* Walker and *Porthesia scintillans* Walker, feeding on castor in India (Muthukrishnan and Senthamizhselvan, 1987; Senthamizhselvan and Muthukrishnan, 1989), *Cotesia flavipes* and *Cotesia sesamiae* have been collected only from noctuid and pyralid hosts feeding on gramineous plants (Polaszek and Walker, 1991). This specificity suggests that plants provide important cues to the searching parasitoids. Similarly, it was reported that taxonomically unrelated hosts feeding on the same species of plants frequently shared the same parasitoids (Picard and Rabaud, 1914) and that the amount of parasitism suffered by a polyphagous host species may often depend on the food plant attacked (Vinson, 1981, 1985; Nordlund et al., 1988).

Cotesia flavipes and *Cotesia sesamiae* preferred odors from infested plants over odors from uninfested plants, indicating that both parasitoids were able to identify plants attacked by their hosts. Similar findings have been reported in several tritrophic systems [see Vet and Dicke (1992) for a review]. Dicke et al. (1990a,b) reported that Lima bean and cucumber plants infested with two spotted spider mites, *Tetranychus urticae* Koch, emitted a blend of volatiles that attracted the predatory mites *Phytoseiulus persimilis* Athias-Henriot. Turlings et al. (1990a,b; 1991a,b) found that maize plants damaged by the beet armyworm [*Spodoptera exigua* (Hübner)] emitted volatile chemicals that were attractive to the parasitoid *Cotesia marginiventris* (Cresson).

Odors from maize plants infested with two *S. calamistis* were more attractive to *Cotesia flavipes* and *Cotesia sesamiae* than odors from maize plants infested with a similar number of *Chilo partellus* larvae. *Sesamia calamistis* is a larger stemborer and feeds more voraciously than *Chilo partellus*, thereby inflicting greater injury to the plant and producing greater quantities of frass. The quantities of volatiles emitted may be proportional to the amount of plant injury, and that could explain the greater attraction to plants infested with *S. calamistis*. This hypothesis is supported by the fact that a larger number of hosts on a plant increased attractiveness (Figure 6). Similar observations were made when *Cotesia sesamiae* was exposed to odors from plants infested with *B. fusca*, also a large stemborer, and *Chilo partellus* (Figure 7). In a related study, Potting

et al. (1993) found that odors from maize plants infested with two larvae of *B. fusca* were more attractive to *Cotesia flavipes* than odors from maize plants infested with two larvae of the smaller *Chilo partellus*. These results strongly suggest that the two parasitoids are not able to discriminate between maize plants infested by the four herbivore species under study and that they both seem to use a similar set of stimuli to locate their hosts. In contrast, *Cotesia marginiventris*, which is a generalist parasitoid of numerous species of leaf-feeding insects on a wide range of plants, is able to distinguish between odors of two different plants fed on by the same host and also between two different host species feeding on the same plant species (Turlings et al., 1989). Different herbivore species may cause a differential release of plant compounds that can be detected by parasitoids after experience (Turlings et al., 1990a). For *Cotesia flavipes*, however, learning has not been detected (Potting et al., unpublished data), possibly because the insects live for only 2–3 days (Moutia and Courtois, 1952; Gifford and Mann, 1967; Kajita and Drake, 1969; Potting et al., 1995b). Because of their short life-span, females of *Cotesia flavipes* may search primarily within the habitat where they emerge and may have adapted to attacking all lepidopterous stemborers occurring in that habitat.

Cotesia flavipes was released in the coastal area of Kenya in 1993. The primary target of this biological control program was the introduced stemborer *Chilo partellus*. However, two other stemborer species, *S. calamistis* and *Chilo orichalcociliellus*, occur in the same ecosystem. During the two cropping seasons (long rains, April–June; short rains, October–December), all three stemborer species coexist in maize fields (Overholt et al., 1994a) and in wild grasses (J.C. Mbapila, unpublished data). In periods between cropping seasons, some stemborers estivate in maize stubbles, while others remain actively feeding in wild grasses, including napier grass, in the proximity of cultivated areas (J.C. Mbapila, unpublished data). The results of this study suggest that *Cotesia flavipes* may be able to search and attack all three stemborer species in cultivated and wild grasses during the cropping seasons and in wild grasses during periods between cropping seasons. Moreover, when maize and sorghum are grown in proximity, our results suggest that *Cotesia flavipes* may exhibit a greater propensity for searching in maize.

Cotesia flavipes and *Cotesia sesamiae* are attracted to odors from the same host habitats, which suggests that the niches of the two parasitoids overlap and competition for hosts is likely to occur. However, due to the differences observed in host-plant preference and host suitability (Ngi-Song et al., 1995), both parasitoids may persist in the same region.

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APPLE FOLIAGE ENHANCES MATING DISRUPTION OF LIGHT-BROWN APPLE MOTH

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Abstract—Uptake and release of pheromone and behavioral inhibitor of *Epiphyas postvittanna* by apple leaves was tested using field electroantennograms (EAG), trap catches to synthetic lures and virgin females, and chemical analysis. Trap catches in single apple trees ($N = 3$) were monitored for six cycles of six days' duration, using delta traps baited with synthetic pheromone. Polyethylene dispensers (0, 1, 10 per tree) releasing pheromone and inhibitor were present for only the first three days of each cycle. Application of 10 dispensers per tree resulted in complete disruption of trapping, which continued for one day after dispensers were removed. Over the three nights following the removal of the dispensers (days 4–6), trap catch was 0, 10, and 15% of the control catch. In contrast, the presence of only one dispenser per tree led to 0–20% of control catches, but on the three nights following dispenser removal catches were 35, 40, and 80% of the control catch. Field EAGs indicated significantly higher relative pheromone concentrations in the trees with 10 dispensers present, compared to trees with single dispensers, but removal of dispensers produced no detectable treatment effect compared to the control trees one day after dispenser removal. In a second experiment, releases of marked male moths into apple orchard plots following the removal of polyethylene dispensers (1 hr earlier that day) resulted in significantly lower catches in traps baited with virgin females in blocks that had been treated, compared to controls. Recovery of pheromone by solvent washing of leaves loaded with 50 μg of the main component of the sex pheromone (1.26 $\mu g/cm^2$) was low (2.5%). Leaves held in a pheromone-saturated atmosphere were loaded with 0.045 ± 0.007 μg pheromone/cm^2. Analysis of apple leaves taken from a pheromone-treated tree at different distances from the pheromone dispenser showed a decay of the pheromone load per square centimeter with increasing distance from the dispenser, as previously indicated by EAG.

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Key Words—*Epiphyas postvittana*, Tortricidae, mating disruption, pheromone, apple leaves, adsorption, release rate, electroantennogram, EAG, light-brown apple moth.

INTRODUCTION

Adsorption and release of insect sex pheromone has been recorded from several plants (Wall et al., 1981; Noldus et al., 1991; Karg et al., 1990, 1994; Sivinski et al. 1994). Wall et al. (1981) and Wall and Perry (1983) showed the possible behavioral importance of this phenomenon in male pea moth (*Cydia nigricana* F.), which precisely located the previous position of a pheromone trap after its removal. Pea leaves functioned as a secondary source of pheromone and release rates were sufficient for male moths to orient to the vacated position of the odor source. The adsorption and release of pheromone by foliage of brassicas more recently has been described by Noldus et al. (1991). Their aim was to investigate the possible role of plants to bridge the gap between the time of calling of females and the searching behavior of parasitoids. Their experiments showed that plants adsorbed the female sex pheromone and released it over an extended period of time. In addition, they showed that male *Mamestra brassicae* L. was attracted to Brussels sprouts (*Brassica oleraceae* L.) releasing the female sex pheromone.

The impact and potential importance of adsorption and release of pheromone by plants on the temporal and spatial distribution of pheromone in mating disruption has long been neglected. However, experiments by Karg et al. (1990) and Karg and Sauer (1992) showed the importance of grape vines for the pheromone concentration achieved in a vineyard treated with pheromone for mating disruption of the European grapevine moth *Lobesia botrana* Hb. In areas with fully developed vegetation, the pheromone concentration was more than 100-fold higher than the same location without foliage in spring or in a defoliated vineyard nearby (Karg and Sauer, 1992, 1995). In addition, the plant canopy affects the structure of the odor plume, with pheromone more evenly distributed in vineyards with a dense plant canopy. The EAG signal shows more intermittency without vegetation. We reported greater variance of EAG signals recorded above grass, compared to adjacent apple tree rows (Suckling et al., 1994). Bengtsson et al. (1994) also showed that pheromone concentrations were lower above a pea canopy and the EAG signal fluctuated more above than within the canopy, although the signal variance was not examined in detail.

Measurements of the adsorption and release of the pheromone of *Epiphyas postvittanna* (Walker) showed the effect of apple leaves in more detail (Karg et al., 1994). Pheromone released from apple leaves was still detected 24 hr after exposure of the leaves to pheromone. Traps baited with leaves exposed to pheromone caught significant numbers of males for three nights after treatment. EAG

measurements along a transect through a pheromone-treated orchard showed significant differences in the EAG response and therefore in the mean pheromone concentration, with increased concentrations closer to the trees in which the dispensers were present. These results were interpreted as showing that leaves could affect the atmospheric pheromone concentration and its temporal distribution and could thereby enhance the success of mating disruption.

In this study, we hypothesized that the release of pheromone from foliage would be sufficient to disrupt male behavior. Experiments were carried out in order to examine the impact of pheromone adsorption and release on apple trees. In addition, the adsorption by apple leaves of the pheromone and inhibitor of *E. postvittanna* was quantified by chemical analysis.

METHODS AND MATERIALS

Insects. Male and female *E. postvittana* were obtained as pupae from a colony in Auckland, New Zealand. The pupae were then kept at 20°C until eclosion, and kept at 12°C for one to two days before use.

Pheromones. Dispensers containing 54.9 mg (*E*)-11-tetradecen-1-yl acetate (*E*11-14:OAc), 2.5 mg of (*E*,*E*)-9,11-tetradecan-dien-1-yl acetate (*E*9,*E*11-14:OAc), 19.7 mg (*Z*)-11-tetradecen-1-yl acetate (*Z*11-14:OAc) as well as 16.8 mg of other substances such as stabilizers, were obtained from Shin-Etsu Chemicals Co., Tokyo. This blend has the disadvantage that *Z*11-14:OAc inhibits trap catch when presented with the pheromone (Rumbo et al., 1993). However, this blend has been used very successfully in a great number of mating disruption trials (Suckling and Shaw, 1991, 1995; Suckling et al., 1990a, 1994) and was therefore tested in these experiments.

Traps. Standard delta traps (Suckling and Shaw, 1990) with sticky bases, baited with rubber septa containing 100 μg of *E*11-14:OAc and 5 μg of *E*9,*E*11-14:OAc (Bellas et al., 1983) were used. Traps were checked daily and sticky bases changed every 10 days. To avoid contamination, separate traps were used for controls when dispensers were present, and after dispenser removal, for both dispenser treatments. In the second field experiment, these traps were baited with virgin female moths held in gauze canisters (Suckling et al., 1990b).

Electroantennogram Apparatus. The electroantennogram (EAG) apparatus and measuring procedure used in these experiments are described in Karg et al. (1994) and Suckling et al. (1994) in full. Briefly, the insect antenna was held between wells containing Ringer solution and silver–silver chloride electrodes. The antenna holder was placed inside a glass chamber, protecting the antenna from ambient air, with a steady airstream (ca. 2.5 liters/min) through the chamber transporting odors or charcoal-filtered air to the antenna. The signal was amplified, filtered, and stored on a computer. For calibration purposes, a 15-ml

volume of air was blown by a second pump across a rubber septum and into the main airstream. The rubber septum (Arthur H. Thomas, Philadelphia, Pennsylvania) was loaded with 105 μg of the true pheromone blend as used in the delta traps. Three measurements of antennal responses were averaged and normalized to the responses to three calibration pulses in charcoal-filtered air, giving one reading of relative EAG amplitude. Normalizing was defined as $EAG_{ambient\ air}/EAG_{calibration}$. Normalization was necessary because of differences in antennal sensitivity to pheromone, as well as changes over time in the same antenna. Nine EAG readings were made for each day of each treatment. The normalized EAG values for the control sites were subtracted from those taken at treated sites. This value is defined as the differential EAG amplitude.

Field Experiment 1. The trial site was a mixed cultivar orchard block (5 m between rows, 5 m within row tree spacing), at the Lincoln University Biological Husbandry Unit. This site was chosen due to its spray-free status and a history of high *E. postvittana* catches during past seasons. Three trees in each block (three blocks) were chosen with similar foliage cover, with at least one guard tree between experimental trees. Each tree had three trap sites marked approximately equidistant around the periphery (radius of ca. 1.5 m) at ca. 1.6 m height. Trees were untreated (control) or treated with either one dispenser in the center of the center-leader tree, or one dispenser placed at each of 10 sites (one in the center of the tree and three between each trap site, around the tree periphery). Dispensers were removed after three days. Trap catch was recorded for this period and for the following three days. After this period, the treatments were cycled among the same trees, in the following manner: after three days without dispensers, the trees that had been treated with the one dispenser treatment became the controls, the trees with 10 dispensers received the treatment with one dispenser, and the controls received the 10 dispenser treatment. To minimize effects of the daily variation of moth flight, treatment of each block was staggered one day and each block was used for six cycles. EAG recordings were taken at the trap positions (as described above), after removal of the traps.

Field Experiment 2. The effect of disruption from pheromone and inhibitor released from foliage alone was also examined in the following manner. Dispensers ($N = 36$) as above were placed into orchard plots ($N = 4$, 22.5 $\times$ 22.5 m), consisting of six rows of trees, with 12 trees per row for two days. Comparable blocks ($N = 4$) 60 m away were untreated. Nine delta traps, each baited with three virgin female moths held in a gauze container (Suckling et al., 1990b), were put into a 3 $\times$ 3 array (9-m sides), at 1.5 m height, in order to maximize moth catch in each plot. Traps were placed in alternate trees from those that had contained dispensers in the central three rows (one guard row on one side, and two guard rows on the other). Male moths were anesthetized with CO_2 and marked with felt tip pens (Suckling et al., 1990b). After two days in place,

dispensers were removed from the orchard at 4 PM. At 5 PM, 52–125 marked males were released among four sites in the middle two rows between traps. Moths were released onto a plastic tray, and those incapable of flight were subtracted from the total. Trap catch of wild and marked moths was recorded the next day. This procedure was repeated four times, in a different pairs of treated and control blocks on each night.

GC and GC-EAD. A Hewlett Packard model HP 5830 A gas chromatograph with a DB-Wax capillary column (30 m × 0.25 mm) and a flame ionization detector (FID) was used for chemical analysis. After 3 min at 80°C, the temperature was increased at 10°C/min. up to 230°C. The carrier gas was hydrogen at 140 kPa. A 1 : 1 split separated the outlet of the column between the FID and the electroantennogram detector (EAD). The EAD signal was amplified and filtered according to standard procedure (Hansson et al., 1989) and printed with a chart recorder at a chart speed of 33 mm/sec. The FID signal was recorded with a HP 18850 A GC-Terminal integrator.

Experiments were carried out in order to identify the GC peaks representing pheromone and inhibitor *E*11–14:OAc and *Z*11–14:OAc, respectively, and *n*-decan-1-ol (11:OH) (which elicits no EAD response). These compounds were independently injected in order to measure their respective retention times. For the analysis of the leaf extracts, 6 μl of the solvent extract was injected. All GC-EAD experiments were repeated at least three times. The low quantity of diene present in dispensers prevented its inclusion in these experiments, due to difficulties in obtaining reproducible results near the detection threshold.

Topical Application of Pheromone onto Leaves. Five micrograms of *E*11–14:OAc (50 μl *n*-heptane containing 100 ng/μl *E*11–14:OAc) was applied to 15 leaves (of similar age and an approximate size of 40 cm^2), and left for 30 min to be adsorbed by leaf waxes, before being put into a polyethylene bag. The same number of untreated control leaves were in a second plastic bag. After 14 hr, the apple leaves were taken out of each plastic bag and washed in 50 ml solvent (25 ml redistilled *n*-heptane and 25 ml redistilled *n*-hextane) for 14 hr on an orbital shaker at 150 rotations/min. The extract was then filtered twice using analytical filter paper (Munktells, Sweden, type 1002). As an internal standard, 5 μl 11:OH was added to the extract. The volume of the extract was reduced to 10 ml under nitrogen, and 6 μl of the extract was injected into the GC for both GC and GC-EAD analysis.

Pheromone Loading of Leaves at Saturation. Three small branches with six to seven apple leaves were taken from an untreated orchard, the cut stems placed in a vial containing water, and the branches taken to the laboratory. There each branch was placed in a 20-liter plastic bag each containing a small fan generating an intermittent airstream at 0.25 m/sec (cycling on and off every 30 min) for 14 hr. The plastic bags were sealed for separation from the outer

environment. In the first plastic bag, four aged dispensers with an average liquid length of 148 mm were attached to a metal frame inside the sealed plastic bag. The mean release rate of each dispenser, estimated by liquid length (Bradley et al., 1995), was 23.7 μg per hour. It was ensured that there was no physical contact between the dispensers and apple leaves. The same number of untreated control leaves was placed in a second plastic bag. After 14 hr, the apple leaves were taken out of each plastic bag and treated as above.

Pheromone Loading of Leaves Taken from Treated Fields. Ten aged pheromone dispensers with a mean liquid length of 146 mm were applied to 10 mature apple trees on May 18, 1994, at one per tree. After 39 days, leaves at different distances from the dispensers were picked and treated as described above. Leaves were taken at approximately 0.04 m, 0.3–0.4 m, and 0.9–1 m away from a central dispensers. As a control, 15 leaves were taken from an untreated orchard. Leaves were washed and the pheromone extracted as above.

Calculation of Amount of Pheromone Uptake by Leaves. The size of all leaves used in these experiments was measured. Leaves of similar size and age were used as far as possible, and the amount of pheromone per square centimeter of leaf area was calculated by dividing the calculated amount of pheromone by the sum of the leaf areas. The amount of pheromone taken up by the leaves was calculated using the internal standard (50 μg 11:OH) as follows: amount of pheromone = $(\text{area}_{\text{pheromone}} \times 50\ \mu\text{g})/\text{area}_{\text{internal standard}}$.

Statistical Analysis. In the first field experiment, the catches of moths in traps were tested for fit to the normal distribution and found to be skewed. Catches were then compared between treatments using analysis of variance and *t* tests of the log transformed total from three traps per tree and back-transformed for presentation (by anti-logs). Catches were pooled from each tree to avoid pseudoreplication (Hurlbert, 1984). The three blocks and six repetitions through time provided 18 replicates. In the second field experiment, trap catches in all nine traps per plot were again pooled because traps in each plot were not true replicates. Recapture rates were compared between treatment and control plots using Fisher's exact test (Zar, 1984), and catches of wild moths were compared by *t* test after log transformation. The analysis was performed using individual nights as replicates. EAG values were normally distributed and were compared untransformed using analysis of variance and *t* tests.

RESULTS

Field Experiment 1, Trap Catches. No moths were caught in trees treated with 10 dispensers while the dispensers were present (Table 1). The catch was also significantly suppressed for three days after removal of the dispensers, compared to the controls ($t = 2.36$, 16 *df*, $P < 0.05$). Trap catch for the three

TABLE 1. BACK-TRANSFORMED MEAN CATCH PER TRAP PER NIGHT (SEM) OF MALE *Epiphyas postvittana* IN PHEROMONE TRAPS PLACED IN SINGLE APPLE TREES WITH OR WITHOUT POLYETHYLENE DISPENSERS RELEASING PHEROMONE AND INHIBITOR[a]

	Dispensers in tree			Dispensers removed		
Treatment	Night 1	Night 2	Night 3	Night 4	Night 5	Night 6
Control	0.30 (0.20)	0.26 (0.02)	0.21 (0.12)	0.24 (0.10)	0.30 (0.05)	0.54 (0.26)
1 dispenser	0.04 (0.04)	0 0	0.04 (0.04)	0.08 (0.06)	0.17 (0.08)	0.49 (0.25)
	(*)	*		*		
10 dispensers	0	0	0	0	0.04 (0.04)	0.04 (0.08)
	*	*	*	*		

[a]Significance from the control is indicated by: (*) for $P < 0.10$ or * for $P < 0.05$.

nights after dispenser removal was 0, 10, and 15% of the control catch (Figure 1). Trees treated with a single dispenser also had suppressed trap catch during the presence of dispensers. Single moths were caught on two occasions, compared to 25 moths in the controls, so that catch in this treatment averaged 4% of catches in control trees. After removal of the single dispenser, trap catch subsequently increased over the following nights to 33, 40, and 80% of the control catch (Figure 1), with no significant difference from control catches (Table 1).

Field Experiment 2. Moth catches were significantly reduced in blocks that had been treated with pheromone up to 1 hr before moth releases (Table 2). The mean level of recapture in the control was 28% (SEM 2.5%), with 10.3% (2.1%) recapture in the previously pheromone-treated plots. Hence, the pretreatment of foliage with pheromone resulted in a reduction to 37% of the catches from that in the untreated plots. This level was very similar to the result on the first night of dispenser removal in the previous experiment (35%, Figure 1). Catch of wild moths was also reduced to 35% in previously treated [mean of 6.3 (SEM1.5)], compared to control plots [mean of 17.5 (SEM 5.1)] ($t = -2.43$, $df = 6$, $P = 0.05$).

Field Experiment 1, EAG Measurements. Differential EAG measurements at the periphery of the trees (after the removal of traps) reflected the presence

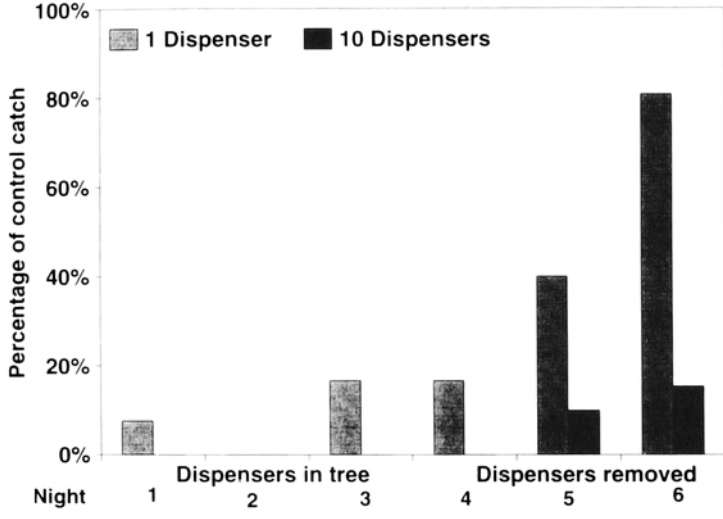

FIG. 1. Percentage of trap catch of male *Epiphyas postvittana* in apple trees, recorded for three nights during pheromone and inhibitor application and for three nights after removal of the polyethelene dispensers.

TABLE 2. CATCHES OF MARKED AND WILD MALE MOTHS IN ONE NIGHT IN APPLE ORCHARD PLOTS, TREATED OR NOT WITH PHEROMONE AND INHIBITOR RELEASED FROM POLYETHYLENE DISPENSERS FOR TWO DAYS UP TO ONE HOUR BEFORE MOTH RELEASES

	No. released		Laboratory-reared moths[a]			Wild moths	
Test	Treated	Control	Treated	Control	P^b	Treated	Control
1	125	119	7(6%)	28(24%)	0.02	9	32
2	116	119	15(13%)	32(31%)	0.01	2	8
3	117	114	19(16%)	38(33%)	0.01	7	15
4	52	57	3(6%)	14(25%)	0.07	7	15

[a]Percentage recapture for marked moths in parentheses.
[b]Fisher's exact test.

of high concentrations of atmospheric pheromone with one or 10 dispensers in the trees (Figure 2), although readings in the treated trees were only significantly higher than the control trees on the second day ($P < 0.05$). EAG values declined after removal of the dispensers, with no differences from control levels after 24 hr. The comparatively insensitive field EAG response in this species is due to

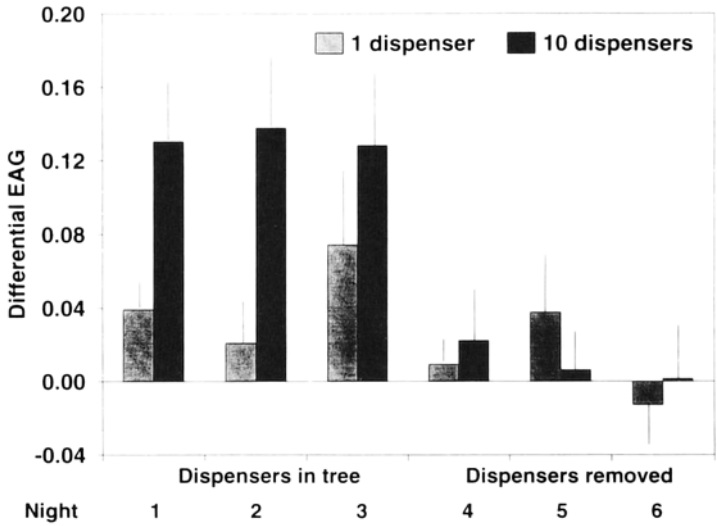

FIG. 2. Differential electroantennogram amplitudes recorded in trees during the presence or after removal, of one or 10 polyethylene dispensers containing pheromone and inhibitor. Bars show the standard error.

the high background response to environmental odors (Karg et al., 1994; Suckling et al., 1994; Rumbo et al., 1995).

GC-EAD Analysis. GC analysis of the extract of the untreated leaves did not show a GC peak with a retention time corresponding to those measured for *E*11-14:OAc or Z11-14:OAc (Figure 3). There was also no antennal response from the male. *E*11-14:OAc as well as Z11-14:OAc were present in the extract of saturated leaves as well as in the extracts of the leaves taken from the field

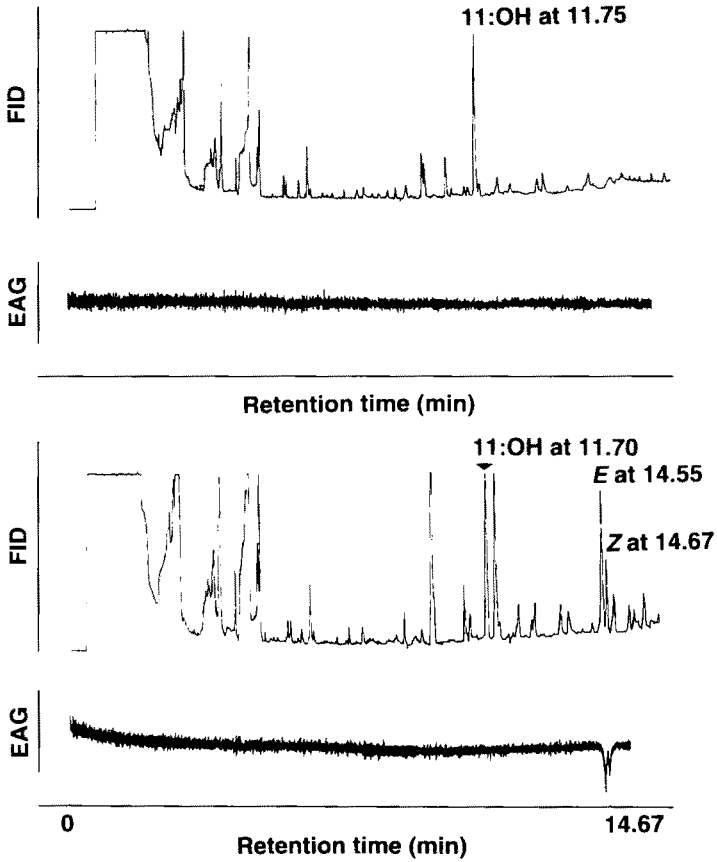

FIG. 3. Gas chromatogram and coupled electroantennogram (EAG) recordings of antenna of male *E. postvittana*. The upper traces show a chromatogram from a untreated leaf and the corresponding EAG recording. The lower traces show the chromatogram of pheromone saturated leaves and the corresponding EAG recording. Pheromone and inhibitor peaks are indicated by arrows.

at different distances from the dispenser. A typical GC-EAD recording for leaves exposed to the dispensers is shown in Figure 3. Antennal response was elicited by *E*11–14:OAc and Z11–14:OAc (as reported by Bellas et al., 1983).

Chemical Analysis of Extracts From Treated Leaves. GC analyses detected that 0.032 ± 0.004 μg of *E*11–14:OAc/cm^2 leaf area could be recovered from topically treated leaves averaging 39.6 cm^2. Hence 1.26 μg of *E*11–14:OAc/cm^2 of leaf area had an aquisition rate of 2.54%. No pheromone was found on untreated leaves.

Chemical Analysis of Extracts from Saturated Leaves. The chemical analysis detected 0.045 ± 0.007 μg of *E*11–14:OAc and 0.019 ± 0.002 μg of Z11–14:OAc (average leaf area of 49.8 cm^2) taken from a bag containing the four dispensers (Table 3). This is a 1:0.42 ratio of *E* to *Z* in the leaves from a saturated environment, compared to a 1:0.42 ratio in dispensers, indicating no error in the ratio of the two components and, therefore, more precision in these data than was evident from the standard errors. Comparison with the results from the treated leaves suggests that ca. 1.66 μg/cm^2 of *E*11–14:OAc was loaded in the saturated air. No pheromone was found on untreated leaves.

Leaves Taken from a Pheromone-Treated Orchard. Analysis of the leaves taken from the field at different distances from the pheromone dispensers showed the highest load on leaves taken close to the dispensers. At 4 cm from the source of pheromone, leaves contained an estimated 0.014 ± 0.009 μg of *E*11–14:OAc and 0.004 ± 0.004 μg of Z11–14:OAc. The *E*:*Z* ratio was 1:0.26, compared to a ratio of 1:0.43 at 30 cm, and 1:0.39 at 95 cm from the dispenser. This comparison implies less precision in the data for the point nearest the pheromone dispenser, which is corroborated by the larger error bars (Figure 4).

The amounts of detectable pheromone and inhibitor decreased linearly with the square root of the distance from the dispenser, so that almost no pheromone was detectable in foliage about 1 m from the source (Figure 4). The change in concentration of *E*11–14:OAc = 0.0169 − 0.0016*x* (where *x* = square root of

TABLE 3. LOAD OF *E*11–14:OAc AND Z11–14:OAc (PHEROMONE AND INHIBITOR) ON LEAVES TOPICALLY DOSED WITH *E*11–14:OAc OR FROM A SATURATED ENVIRONMENT WITH A 70:30 RATIO

Treatment	Mean load of (μg/cm^2) *E*11–14:OAc	Standard deviation	Mean load of (μg/cm^2) Z11–14:OAc	Standard deviation
Topically dosed	0.032	0.004	NA	
Saturated	0.045	0.007	0.019	0.002

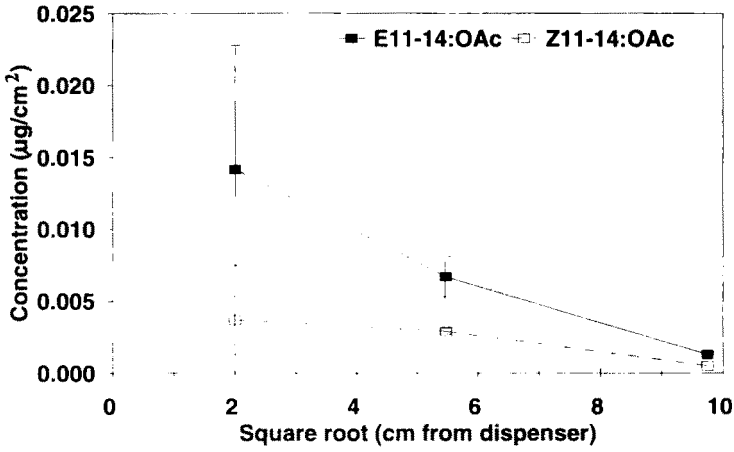

FIG. 4. Decay of the pheromone and inhibitor load on apple leaves with square root of distance from a dispenser.

distance from dispenser in centimeters, $r^2 = 0.61$, $df = 8$, $P < 0.05$). The change in concentration of Z11-14:OAc = $0.0048 - 0.0004x$ (where x = square root of distance from dispenser in centimeters, $r^2 = 0.34$, $df = 8$, $0.10 < P < 0.05$). Leaves taken only 4 cm from a dispenser were far below saturation with either E11-14:OAc and Z11-14:OAc. The estimate of proportion of saturation of these leaves compared to those kept in a saturated bag varied from 0.2 (Z11-14:OAc) to 0.3 (E11-14:OAc).

DISCUSSION

Trap Catches. Several possible explanations for the initial reduction in trap catch after removal of the dispensers can be identified. First, the pheromone and inhibitor released from the apple leaves may have masked the pheromone plume released from the traps, decreasing their attractiveness. Although this species discriminates spacially separated pheromone and inhibitor sources (Rumbo et al., 1993), the pheromone component being released from the foliage may have masked the more directional plume of pheromone from the rubber septa in the traps, reducing the peak-to-mean ratio (Murlis et al., 1992). Second, adaptation and habituation of the sensory system of insects in the vicinity of the trees that had previously held dispensers could have been caused by the background of pheromone released from the leaves, reducing male responsiveness to pheromone released by traps. In addition, male responsiveness could be reduced to inadequate stimulation with pheromone. The leaves in the treated

trees would continuously release pheromone, thereby generating a comparatively stable pheromone background. This type of pheromone cloud arrests the upwind flight of male moths approaching a pheromone source (Kennedy et al., 1981), because intermittent pheromone stimulation is required for sustained upwind flight (Baker et al., 1985). However, decreased trap catches over one to two days cannot be explained by those phenomena alone.

Recovery of preexposed males from habituation may be quite rapid, on the order of minutes (Rumbo and Vickers, unpublished data) or perhaps hours (Bartell and Lawrence, 1973), although this is likely to be dose-dependent. Given the extent of moth movement and the physical layout of the trees in small blocks, it seems very unlikely that most or all moths in the vicinity of the treated trees would be disrupted for three days after removal of dispensers. There was no evidence that catch in the controls was affected by the presence of the dispensers in nearby trees.

However, the suppression of catch indicates that leaves released enough pheromone in the first night after dispenser removal to affect male orientation in single trees. This effect may be substantially more influential in orchards without the large untreated areas that characterize the field site used in the first experiment here. This effect was further tested using larger plots in the second experiment, where release of moths not previously exposed to pheromone ensured that the only source of pheromone and inhibitor affecting catch to female-baited traps was from the foliage. In this case, the effect was significant for each test. The results from wild moths also closely corroborated the first experiment.

EAG Measurements. The EAG was capable of detecting a significantly higher atmospheric pheromone level when 10 dispensers were present in each tree, compared to the presence of single dispensers or no dispensers. However, traps more effectively displayed a difference in efficacy of disruption between treatments. The sensitivity of the EAG readings was insufficient to detect any effect from the pheromone treatment on the day after the removal of the 10 dispensers. Higher mean EAG values in the treatments with 10 dispensers present were mainly due to detection of intermittent peaks arriving from nearby dispensers, rather than a noticably raised background, and this effect is evident in the higher standard errors of EAGs recorded in trees with 10 compared to one dispenser.

Chemical Analysis. Our experiments confirm that the major pheromone component and inhibitor of *E. postvittana*, *E*11-14:OAc and *Z*11-14:OAc, are taken up and released by apple leaves. Hrdý et al. (1990) used GC-EAD to confirm the uptake of pheromone by stonefruit tree foliage, using Oriental fruit moth (*Cydia molesta* Busck). Uptake is presumably due to the similarity of the chemical attributes (lipophilicity) of the pheromone and the waxes (Jeffree, 1986; Baker, 1982).

The velocity and direction of the process could be determined by the pher-

omone gradient, the volume of the wax, as well as the wind speed and pheromone concentration of surrounding air. the small proportion of pheromone recovered from the leaves can be explained partly by this shift of the equilibrium towards the airborne pheromone with increased release of the phermone into the surrounding air, compared to the uptake, that takes place simultaneously. Adsorption of pheromone onto the glass walls remains a technical problem for the technique used, as it decreases the amount of pheromone recovered. The low proportion of pheromone recovered from leaves harvested from the field, compared with the saturated environment, was likely to be due to the lower ambient concentration.

Airborne pheromone of *E. postvittana* released from polyethylene dispensers is taken up within seconds by apple leaves (Karg et al., 1994). The decline in pheromone load with distance corroborates the EAG measurements of Karg et al. (1994), where the EAG response to pheromone from leaves was inversely related to the square root of distance from a central pheromone source. Unlike the first experiment, where the pheromone was directly applied to the waxes covering the leaves, the pheromone had to be taken up from the surrounding orchard air. The amount of pheromone taken up depends on the airborne pheromone concentration and on the partition constant of the pheromone into the leaf-waxes. Factors limiting the uptake include atmospheric pheromone concentration, length of time of exposure to pheromone in moving air in the field, and amount and chemical constitution of leaf waxes. The amount and composition vary with leaf age and season, and different varieties may have different waxes (Fernandes et al., 1964). In our experiment, the quantity of *Z*11–14:OAc released from dispensers and detected from foliage was small, accounting for the lower level of significance compared to *E*11–14:OAc.

A static model of pheromone concentrations in orchards, based on diffusion gradient alone, would suggest that concentrations taken up and released by foliage near dispensers are high, with isoconcentration spheres in foliage centered on dispensers. Karg et al. (1994) used EAG readings from leaf transects to show that this model was basically correct. Concentrations of pheromone in the air among foliage would therefore be predicted to have a three-dimensional distribution centered on major (dispenser) and minor point sources (leaves), with rapidly diminishing average concentrations away from the major sources. Instantaneous atmospheric concentrations would also be affected directly by filamentous plumes from dispensers, as well as the more numerous but less concentrated pheromone plumes emitted from foliage. The arrival of odor plumes, or "bursts" with high pheromone concentrations (Murlis and Jones, 1981; Murlis, 1986; Murlis et al., 1992) from these point sources would give much higher instantaneous pheromone concentrations.

Wall et al. (1981) placed wheat leaves near the open ends of pheromone traps containing rubber septa loaded with 10 mg pheromone (*E*8,*E*10–12:OAc),

and used GC and GC-EAD techniques to confirm behavioral observations that the foliage was releasing pheromone. Adsorption and release of pheromone can play an important role in mate-finding, as reported by Noldus et al. (1991) for another moth and by Sivinski et al. (1994) for fruit flies. In the context of mating disruption, the pheromone released from foliage will also assist adaptation and habituation of males by providing a background level of pheromone and lowering the signal-to-noise ratio. This ratio is recognized as necessary for sustained upwind flight (Willis and Baker, 1984), so any reduction in it may lead to a reduction in take-off and subsequent behaviors and consequently mate finding. In our experiments, the pheromone and inhibitor release rates from leaves were sufficient to lead to a reduction in trap catch after removal of the pheromone dispensers for one to two nights.

The results of this study corroborate the findings of earlier studies on pheromone adsorption and release from plants. Crop foliage is likely to be of great importance for the success of mating disruption by increasing the likelihood of unstructured pheromone clouds with reduced variance in concentration and temporal distribution. Apple leaves can function both spatially and temporally as pheromone buffering systems by reducing wind speed, as well as acting as a source for secondary release of pheromone. In our view, uptake and release of pheromone from plant waxes at many point sources (= leaves) generate a background concentration supporting disruption. The relative importance to disruption of leaf-released pheromone and pheromone released directly from dispensers is still uncertain, but these experiments indicate that the role of crop foliage in mating disruption should not be overlooked.

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SEARCH FOR TSETSE ATTRACTANTS: A STRUCTURE-ACTIVITY STUDY ON 1-OCTEN-3-OL IN *Glossina fuscipes fuscipes* (DIPTERA: GLOSSINIDAE)

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Abstract—Trapping tsetse flies belonging to the *palpalis* group still relies totally upon luring by visual cues even though odor-baited trapping is used effectively against the *morsitans*-group species. Forty-three percent of the antennal olfactory cells of *Glossina f. fuscipes*, a member of the *palpalis* group, respond to 1-octen-3-ol. For this species we report a structure–activity relationship between 1-octen-3-ol analogs, in which carbon chain length and the configuration of the hydroxyl and π-bond moieties are varied, and biological activity. Although the optimum chain length for all cells sensitive to 1-octen-3-ol is eight and most cells give lower responses when the hydroxyl function is omitted, there is a clear division into two groups. One group is diverse and represents cells that appear indifferent to the presence or position of the π bond; many will respond to such disparate structures as acetone and 3-methylphenol as well as to 1-octen-3-ol. In the other group, the structural requirements for the stimulus are more stringent; the cells appear to be specifically tuned to 1-octen-3-ol. Their thresholds are three orders of magnitude lower than those of the former group. The existence of two clusters points to a functional division in the olfactory sense. We suggest that the latter low-threshold group is involved in host detection from a distance while the former diverse group is involved in host discrimination at close range. Trap harvests with 1-octen-3-ol as a bait may have been disappointing because the appropriate mixture for generating a landing response on the traps is still lacking.

Key Words—Tsetse, *Glossina f. fuscipes*, host location, olfaction, structure-activity relationship, electrophysiology, 1-octen-3-ol.

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INTRODUCTION

The thirty-one species of tsetse flies (*Glossina* spp.) have been classified by morphology and habitat into three groups: the *morsitans*, *palpalis*, and *fusca* tsetse (cf. Jordan, 1977). All feed exclusively on blood, which they take from mammals, birds, or reptiles (Weitz, 1963). Although host odors lure flies of the *morsitans* group (e.g., Hargrove and Vale, 1978; Owaga, 1984; Torr, 1989; Vale, 1974), the *palpalis* group "gives the impression that it detects its hosts by sight rather than smell" (ex., Laveissière et al., 1990). Indeed, trapping of the *palpalis*-group flies now still relies entirely on providing visual cues (see Cuisance et al., 1991; Gouteux and Sinda, 1990; Gouteux and Le Gall, 1992; and Ogwal et al., 1992 for recent examples).

Chemical baits that attract the *morsitans*-group tsetse include 1-octen-3-ol (Hall et al., 1984), a blend of phenols (Hassanali et al., 1986; Owaga et al., 1988; Vale et al., 1988), and acetone (Vale and Hall, 1985). These are sensed by the antennae. Screening spike responses of olfactory cells to the array of attractants shows that in the *morsitans*-group species *G. m. morsitans*, 40% of the olfactory cells respond to 1-octen-3-ol, 18% to the phenols, and 10% to acetone. A similar survey of the receptors in the *palpalis*-group species *G. f. fuscipes* gives a surprising result: the percentages are 43 for 1-octen-3-ol, 18 for the phenols, and 9 for acetone. Moreover, the sensitivity of the receptors for these odors appears to be the same in the two species (Den Otter and Van der Goes van Naters, 1992; Van der Goes van Naters, unpublished). Thus, electrophysiological experiments suggest that the organization of the olfactory sense in the two is similar even though field trials with odors indicate that the *morsitans*- and *palpalis*-group tsetse behave quite differently.

Clearly, our understanding of the sensory basis of host location in the *palpalis*-group tsetse is still limited. Reexamination of the odor spectra of the antennal olfactory receptors, of which cells responsive to 1-octen-3-ol constitute the largest set, is needed. Our objective in this structure–activity relationship (SAR) study on octenol-derived chemicals is to characterize the specificity of olfactory receptors in *G. f. fuscipes* and to theorize on their role in host location.

METHODS AND MATERIALS

Insects. Pupae of *G. fuscipes fuscipes* Newstead 1910 were obtained from the colony of the Département d'Elevage et de Médecine Vétérinaire (CIRAD-EMVT) in Montpellier, France. The pupae were kept individually in glass vials until emergence of the flies, whereupon these were grouped in cages by sex and age. Pupae and flies were kept in a 12L:12D photoperiod at 25°C and 75%

relative humidity. Flies were fed off rabbits' ears on alternate days beginning the day after emergence.

Electrophysiology. Recording of action potentials was accomplished as described by Den Otter and Van der Goes van Naters (1992). A fly was tested when between 4 and 15 days old; it was fed the previous day. Cells at two sites on the antennae of each fly were tested, one on the medial funicular face and the other on the lateral face. A current of air, issuing from a 7-mm-ID tube at 2 m/sec, was directed at the head of the fly. The tube was provided with an opening through which test compounds were injected.

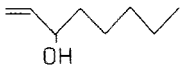
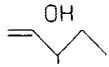
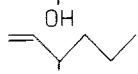
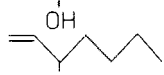
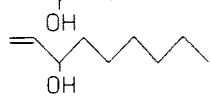
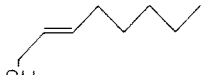
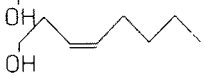
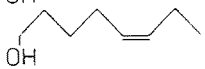
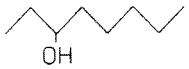
Test Compounds. Test chemicals used included 1-octen-3-ol and structurally related compounds in three sets of experiments (Table 1). These chemicals vary in carbon chain length and in the positions/presence of the π bond and hydroxyl moieties. Additionally, acetone and 3-methylphenol were tested. Purity according to specification was >98% and chiral substances were racemics unless otherwise indicated. All were purchased from Fluka (Buchs, Switzerland) except for the three isomers 2-, 3-, and 5-octen-1-ol (Johnson Matthey, Karlsruhe, Germany), 1-hexen-3-ol (Aldrich, Milwaukee, Wisconsin), and 1-nonen-3-ol (ICN, Cleveland, Ohio). These chemicals are liquids at room temperature and pressure (RTP: 298K, 1 atm). Saturated vapor concentrations at RTP, as determined by weight loss over 24 hr of samples in glass-stoppered 1-liter flasks, are given in Table 1.

Three log step dilutions in air were prepared by transferring appropriate amounts of the saturated vapors of the chemicals in 1.2-liter glass serum bottles fitted with screw caps and rubber seals. Saturation of the atmosphere in a "mother" bottle was achieved and maintained throughout the study by addition of 2 ml of a chemical. The three dilutions from these were remade at the beginning of each day; two dilution series of bottles for each substance were alternated. Thus a bottle used on one day was left standing uncapped the next, the cap and seal being placed on its twin. This allowed the uncapped bottle to cleanse of odor while the rubber seal, after a number of exchanges, had absorbed what it could and was no longer a biasing factor on the concentration of a dilution.

Test chemicals were drawn from the bottles through the rubber seals into plastic syringes (Omnifix 5 ml) immediately before presentation. Syringes were equilibrated with the bottle atmosphere by pumping the plunger 10 times prior to taking a sample. Not more than 10 syringe fills were taken from a bottle per day. A syringe was mounted on a spring-powered device and 1.5 ml of its contents was injected in 0.1 sec into the airstream directed at the head of the fly.

Vapors of acetone and 3-methylphenol were not taken from bottles but were dispensed from filter papers placed inside syringes. Syringes with acetone

TABLE 1. CHEMICALS USED IN EXPERIMENTS 1–3[a]

	Name	Structure	Saturated vapor conc.[b]
	1-octen-3-ol		7.6×10^{-6} M
1)	1-propen-3-ol		1.6×10^{-3} M
	1-buten-3-ol		7.6×10^{-4} M
	1-penten-3-ol		4.5×10^{-4} M
	1-hexen-3-ol		1.4×10^{-4} M
	1-hepten-3-ol		6.4×10^{-5} M
	1-nonen-3-ol		7.1×10^{-6} M
2)	2(*E*)-octen-1-ol		3.2×10^{-6} M
	3(*Z*)-octen-1-ol		3.3×10^{-6} M
	5(*Z*)-octen-1-ol		3.5×10^{-6} M
3)	octane		7.9×10^{-4} M
	1-octene		7.6×10^{-4} M
	3-octanol		7.5×10^{-6} M

[a]The parent compound 1-octen-3-ol was the reference chemical in each experiment.
[b]At RTP: 298K, 1 atm.

contained 10 mg of the chemical; those with 3-methylphenol were charged with 5 mg.

Experimental Procedure. Cells on the antennae were screened for response to a stimulus of saturated 1-octen-3-ol vapor. When a cell sensitive to 1-octen-

3-ol was located, the test chemicals were drawn from the bottles and injected in ascending concentration. The order in which substances were presented was varied. We observed intervals of 30 sec between presentations to allow the cells to return to baseline activity. After all concentrations of each substance in an experiment had been presented, the odor spectra were further characterized with acetone and 3-methylphenol.

Analysis of Data. Recordings were transferred from tape to paper for analysis. The magnitude of response was calculated from the maximum number of action potentials in a space of 0.1 sec after injection of the stimulus, the non-stimulated activity prior to presentation of the odor being similarly measured and subtracted. This figure was multiplied by 10 to obtain spikes per second as the unit of response.

To characterize the odor spectra of the olfactory receptors, multidimensional graphs were envisaged with each axis representing the response to a different substance relative to that on stimulation with 1-octen-3-ol. Here we present graphs with two dimensions only, as more are difficult to visualize. Cells with similar spectra form clusters.

As a supplement to this common-sense visual approach for grouping cells, several types of multivariate statistical analyses were considered, including hierarchical cluster analysis, factor analysis, and multidimensional scaling. Bieber and Smith (1986) note that for cluster and factor analysis "the popularity of these intuitive procedures is rooted in the absence of sound statistical theory concerning the appropriateness of the final solution." Multidimensional scaling has the characteristic that the meaning of the dimensions that reveal the underlying structure in the data is abstract. We have opted for the first of the three (hierarchical cluster analysis from SPSS/PC$^+$ with average linkage over Euclidean distances) and base our choice of cluster solution on a scree procedure.

The different clusters that emerged from the analyses were considered separately, and the average responses of the constituent cells are plotted in dose-response curves. Points in the curves are connected by line segments; sigmoid or other curve fitting did not appear feasible in our graphs due to the limited number of concentrations per substance. This has a drawback: statistical analysis of the dose-response curves is not possible without a function to fit to the data because the concentrations used are different for each chemical. Therefore conclusions are based on the most salient features of the data only.

RESULTS

The results comprise a data set of 183 cells recorded from 41 female flies and 52 males. Responses to four concentrations of 1-octen-3-ol did not differ between the sexes (ANOVA, $P = 0.84$); hence the data are pooled. The three experiments (Table 1) are treated separately.

Effect of Chain Length. The relative effectiveness of the six homologs of 1-octen-3-ol is the same for all 28 cells tested, as is reflected by the graph in Figure 1a. Figure 1a shows a single cluster in a plot with the relative responses to 1-hepten-3-ol and 1-nonen-3-ol on the axes. This is true also in graphs with

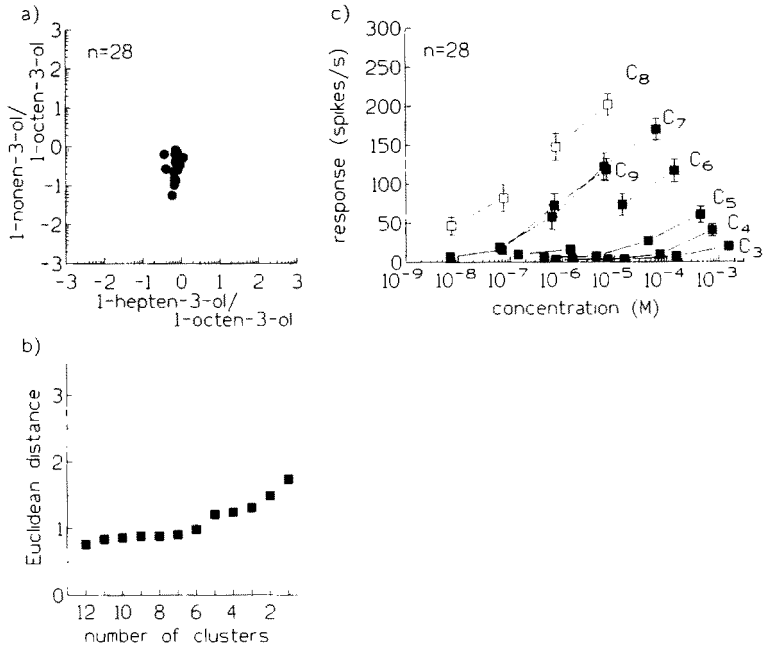


FIG. 1. (a) Spike responses of cells on stimulation with 1-hepten-3-ol (abscissa) and 1-nonen-3-ol (ordinate) relative to the responses on stimulation with the parent compound 1-octen-3-ol. Each data point represents one cell. The plotted values are proportions, have no units, and are averages over four concentrations. Axes are numbered with exponents and extend from 10^{-3} to 10^{3}. Stimuli to be graphed on the axes were selected to maximize clustering of cells. The data points are seen as a single cluster. (b) Scree diagram for the last 12 agglomeration stages in the hierarchical cluster analysis on the 28 cells. Dimensions in the analysis are the relative responses to the six homologs of 1-octen-3-ol as in (a). The ordinate gives the distance over which the two nearest clusters are combined to create the number of clusters given on the abscissa. Each successive stage fuses two clusters that are further apart. The rise is steady and the distances are small as compared with Figure 2b. We choose the one-cluster solution. (c) Dose–response curves for 1-octen-3-ol (C_8) and its homologs. The abscissa is the molar concentration of the stimulus; the ordinate gives the average spike responses with SEM of the cells in the cluster of (a).

any other combination of the homologs represented (not shown). When cells are grouped by hierarchical cluster analysis, the Euclidean distances over successive stages of the agglomeration process show a steady increase (Figure 1b): we allow clustering to progress to its end. In the sample, five cells responded to acetone, three to 3-methylphenol, four to both, and 16 to neither.

Figure 1c shows the average responses to the saturated vapors and three log step dilutions of each homolog. Note that the curves are staggered on the abscissa: the saturated vapor pressures decrease with increasing chain length (Table 1). The C_7 and C_9 curves overlap. A chain length of eight carbon atoms is clearly optimal in the range of homologs tested.

Effect of Relative Positions π-Bond and OH Moieties. When the relative responses to 2-octen-1-ol and 3-octen-1-ol are represented on the axes, two diffuse clusters are apparent (Figure 2a: arbitrarily designated P and Q). These represent 19 and 32 cells, respectively. The scree procedure confirms that a two-cluster solution is appropriate (Figure 2b).

Recordings from the cells in cluster P always showed two spike amplitudes, which suggests that they are collocated with a second type of cell (Figure 4). Further investigation proved the latter responds to 3-methylphenol only and not to any of the other substances tested. Cluster P cells themselves are unresponsive to either acetone or 3-methylphenol. The dose-response curves show that the cells are very discriminating to deviation of the positions of the π-bond and hydroxyl moieties (Figure 2c). The 1-3 configuration is the only adequate structure.

Of the cells in cluster Q, 8 responded to acetone, 2 to 3-methylphenol, 10 to both, and 12 to neither. The curve of 1-octen-3-ol (Figure 2d) lies somewhat below the other three. This suggests that positioning of the hydroxyl group on carbon 1, instead of on carbon 3, may enhance the response. A closer look at Figure 2a seems to bear this out. The points in the cluster are spread over a 45° diagonal relative to the two axes: the variation in the cluster is largely due to the OH-group position rather than to the position of the π bond. Although cells in the left range of cluster Q appear to be indifferent to the OH position (relative responses are around $10^0 = 1$), there is a continuum up to cells that respond 10 times more strongly on stimulation with 2- or 3-octen-1-ol than with 1-octen-3-ol. The cells seem indifferent to the position of the π bond (Figure 2d).

Due to a procedural error, the lowest point in each curve was lost.

Effect of Omission of π Bond or OH. Cluster formation is less pronounced in this experiment (Figure 3a). Several cells are detached from two rather dense agglomerations. This is reflected in the scree diagram by a less salient elbow point (Figure 3b) than is shown in Figure 2b. For ease of interpretation, we chose a two-cluster solution. These are again assigned letters P and Q (38 and 66 cells, respectively).

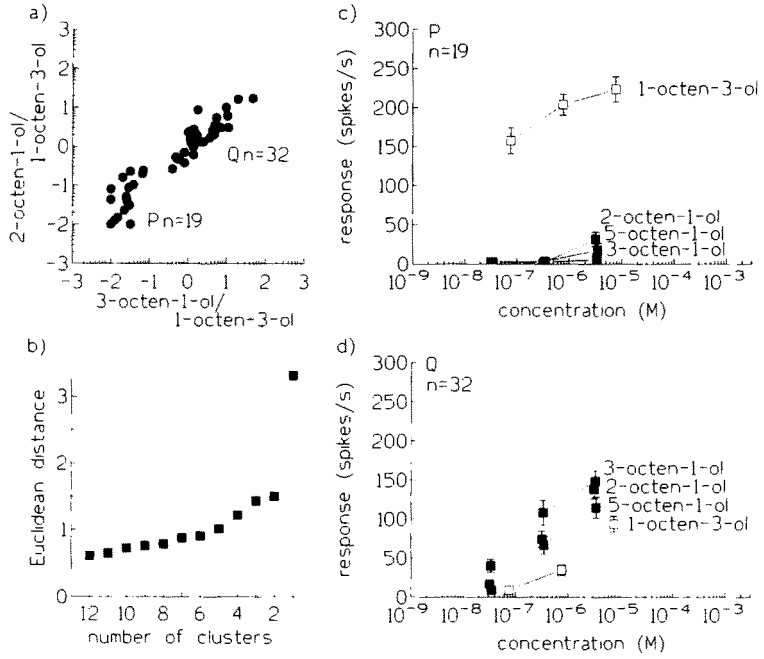


FIG. 2. (a) Spike responses of cells on stimulation with 3-octen-1-ol (abscissa) and 2-octen-1-ol (ordinate) relative to the responses on stimulation with 1-octen-3-ol. See Figure 1a for details. The two clusters are marked P and Q. (b) Scree diagram for the hierarchical cluster analysis by relative response to 2-, 3-, and 5-octen-1-ol. See Figure 1b for details. A salient elbow in the steady rise of Euclidean distances at the two cluster solution suggests agglomeration should be terminated there. (c) Dose–response curves for the cluster P cells in (a). Abscissa: molar concentration of the stimulus; ordinate: average spike responses with SEM. (d) Dose–response curves for the cluster Q cells in (a). Abscissa: molar concentration of the stimulus; ordinate: average spike responses with SEM.

Recordings of cells in cluster P display the characteristics described in Figure 4. The curves in Figure 3c show that 1-octen-3-ol is clearly the most stimulating followed by 3-octanol and 1-octene. Octane gives little or no response.

Cluster Q has a wider spread. In this cluster, 16 cells responded to acetone, 8 to 3-methylphenol, 17 to both, and 25 to neither. The curves for octane and 1-octene overlap, as do the pair for 3-octanol and 1-octen-3-ol (Figure 3d). The latter are clearly more stimulatory on average. However, the averages in Figure 3d mask the extremes. Thus, for a number of cells of the cluster (those on the

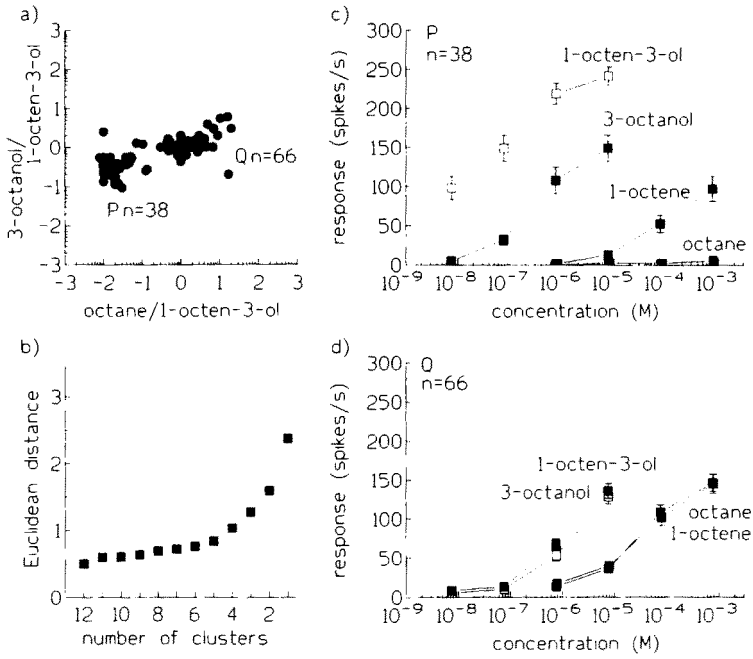


FIG. 3. (a) Spike responses of cells on stimulation with octane (abscissa) and 3-octanol (ordinate) relative to the responses on stimulation with 1-octen-3-ol. See Figure 1a for details. For ease of interpretation, five outliers are disregarded to give two clusters, P and Q. (b) Scree diagram for the hierarchical cluster analysis by relative response to octane, 1-octene, and 3-octanol. See Figure 1a for details. We choose the two-cluster solution. (c) Dose-response curves for cluster P cells in (a). Abscissa: molar concentration of the stimulus; ordinate: average spike responses with SEM. (d) Dose-response curves for cluster Q cells in (a). Abscissa: molar concentration of the stimulus; ordinate: average spike responses with SEM.

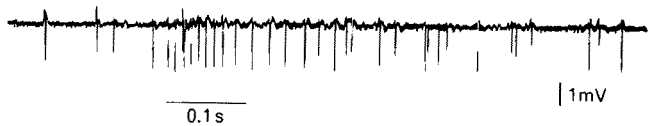


FIG. 4. Response of a cluster P cell (large-amplitude spikes) to a low stimulus intensity of 1-octen-3-ol (0.1-sec duration). Cluster P cells continue firing at an elevated rate beyond stimulus duration. They are insensitive to acetone and 3-methylphenol. Collocated always is a phenol-sensitive cell with similar temporal characteristics (small-amplitude spikes).

right-hand side in Figure 3a), the responses to octane and 1-octene are 10 times those to 1-octen-3-ol.

DISCUSSION

The sample of 183 cells is a motley conglomeration. An underlying order can, however, be made apparent even though our tools are crude. The grouping of cells here is not an expression of our human inclination to classify but is a real feature of the data set. The existence of clusters in the population of cells responsive to 1-octen-3-ol points to a functional division in the olfactory sense.

Clustering proves that the population of olfactory cells works with more than one type of receptor molecule. The wide spread of data points within the clusters—especially for clusters Q in Figures 2a and 3a—suggests that even within a subset of the population there are different types. This may extend to the cell membrane, as was shown for the sugar cells of the fleshfly (Shimada et al., 1974). Until we are sure that a membrane harbors but one type of receptor site, we cannot model sites from SAR studies on a cellular level. The most we can do is indicate which moieties in an odor molecule play a role in the odor-membrane interactions.

Clustering also shows the importance of testing the olfactory cells individually. Antennal differentiation necessitates that reception of odors be studied at the sensory level, by single-cell recording if possible. Perception of odors is reflected by the flies' responses in wind tunnels and field trials. Other behavioral assays may give valuable data as well, although they can be difficult to interpret. Saini et al. (1989) reported an SAR study on 1-octen-3-ol in which they assessed the relative perception of odors by counting the number of flies that raise their antennae to a stimulus. They concluded that the chemoreceptors involved may not be very specific and commented on features of the receptor sites. The differences between our results may be a contrast in species—their work is on *G. m. morsitans*—but more likely reflect differing methodologies.

Whether or not cells form disjunct groups in Figures 1a, 2a, and 3a seems to be dependent on the type of chemicals tested. While all cells respond in similar fashion to the homologous series 1-propen-3-ol through 1-nonen-3-ol, they are differentially sensitive to the other 1-octen-3-ol derivatives.

Clusters P in experiments 2 and 3 (Figures 2a and 3a) appear to correspond: cells of both have the characteristics outlined in Figure 4. From this it follows that clusters Q in experiments 2 and 3 (Figures 2a and 3a) must also be identical. That the two clusters P each represent the same proportion of the total, approximately 37% (experiment 2: $19/51 \approx 37.3\%$; experiment 3: $38/104 \approx 36.5\%$), supports this.

The thresholds of cluster Q cells for 1-octen-3-ol are on average between

10^{-8} and 10^{-7} M (Figures 2d and 3d). The position or presence of a π bond in the molecules appears to be of no consequence. The hydroxyl group is important (Figure 3d: the curve for 3-octanol intercepts the abscissa > 1 log unit lower than the octane curve), and the effect of its position on the spike responses varies between nil and a 10-fold factor for the substances tested (Figure 2a). On average, molecules appear more stimulatory with an OH on carbon 1 than on carbon 3 (Figure 2d).

For cluster P cells the structural requirements for a good stimulant appear to be more stringent than for the cluster Q cells. Both the presence of the π-bond and hydroxyl moieties as well as their position in the molecule are important (experiments 2 and 3: Figures 2c and 3c). None of the tested compounds meet the requirements as well as 1-octen-3-ol. Extrapolating the dose–response curves for 1-octen-3-ol in Figures 2c and 3c to the abscissa gives an average threshold concentration of 10^{-12} to 10^{-10} M.

According to the latest data now available (Torr et al., personal communication), an ox exhales 0–0.119 mg 1-octen-3-ol/hr with an average of 0.014 mg/hr. Presupposing a ventilation rate of approximately 5000 liters/hr (Spector, 1956) and with a relative molecular mass of 128.22 g/mol for 1-octen-3-ol, its concentration in cattle breath is 2×10^{-11} M [0.014×10^{-3}/(128.22 × 5000) g/mol/hr/(hr/g/liter)]. Downwind, the odor spreads into a larger volume, although the rate of diffusion is difficult to estimate.

Despite the decline in concentration downwind of an ox, we suggest that cluster P cells are sufficiently sensitive to 1-octen-3-ol to aid in host detection. Furthermore, threshold concentrations between 10^{-12} and 10^{-10} M will be overestimates if the fly applies a more sensitive method of decoding its afferent signals than we have used in our analyses of the recordings. It is clear, however, that the cells will be functioning at the lower limit of their dynamic range. The cells appear to be specifically tuned to 1-octen-3-ol; no structural alteration tested augmented the response nor did structurally unrelated chemicals prove effective.

The function of the cluster Q cells remains open due to their high thresholds. Many of these cells respond to structurally unrelated compounds as well as to 1-octen-3-ol and its derivatives. Either the prime stimulant is not among these or chemicals in mixtures may act synergistically on a cell. The latter possibility is now being investigated.

With dissimilar but overlapping odor spectra, the cluster Q cells could be involved in discriminating the odor mixtures from different hosts (across fiber pattern coding; cf. Erickson, 1963, 1982). We suggest the cluster P cells aid in host detection from a distance while the cluster Q cells are involved in host recognition at close range.

Contrary to the results of the trapping experiments in the field, our electrophysiological experiments indicate 1-octen-3-ol may be a powerful attractant for *G. f. fuscipes*. Sutcliffe (1987) notes that disparate odors mediate successive

stages in the distance orientation of biting flies to their hosts. The odors that attract tsetse appear to be different from those that cause the flies to alight for feeding (Saini et al., 1993). We suggest that although the *palpalis*-group tsetse should be attracted by 1-octen-3-ol in field tests, the trap harvests thus far may have been disappointing because the mixture required for an alighting response has not yet been developed.

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PHEROMONE CHIRALITY OF ASIAN PALM WEEVILS, *Rhynchophorus ferrugineus* (OLIV.) AND *R. vulneratus* (PANZ.) (COLEOPTERA: CURCULIONIDAE)

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Abstract--Production of 4-methyl-5-nonanol, and 4-methyl-5-nonanone by two sympatric Asian palm weevils, *Rhynchophorus ferrugineus* (Oliv.) and *R. vulneratus* (Panz.) suggested that enantiospecificity of either compound could impart species specificity of pheromone communication. Weevil-produced, racemic 4-methyl-5-nonanol and 4-methyl-5-nonanone and their stereoselectively synthesized optical isomers were subjected to gas chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometry (MS) on a chiral Cyclodex-B column. Only the *S,S* stereoisomer of 4-methyl-5-nonanol was EAD active and was produced by both *R. ferrugineus* and *R. vulneratus*. Production and EAD activity of (*S*)-4-methyl-5-nonanone exceeded that of its antipode in both weevils. In field experiments in Java, (4*S*, 5*S*)-4-methyl-5-nonanol and the stereoisomeric mixture were equally attractive. The 4*R*,5*R* stereoisomer was inactive. The corresponding ketone enantiomers neither enhanced nor reduced attraction to (4*S*,5*S*)-4-methyl-5-nonanol. Lack of apparent differences between *R. ferrugineus* and *R. vulneratus* pheromones suggests that synonomy of both weevils should be considered unless other pre- or postzygotic reproductive isolating mechanisms are disclosed in future studies.

Key Words—Coleoptera, Curculionidae, *Rhynchophorus*, aggregation pheromone, pheromone chirality, 4-methyl-5-nonanol and 4-methyl-5-nonanone, Asian palm weevils.

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INTRODUCTION

Pheromone specificity contributes to habitat and resource partitioning among competing species in the Coleoptera. Only rarely has intraspecific attraction and interspecific interruption of response been attributed to enantiospecific production of more than one aggregation pheromone. (+)-Ipsdienol and (−)-ipsenol produced by *Ips paraconfusus* (Lanier) interrupt the aggregation of sympatric *I. pini* (Say) (Birch et al., 1977, 1980; Light and Birch, 1979). *Trogoderma granarium* (Everts) uses the (*R*)-(−)-enantiomers of (*Z*)- and (*E*)-trogodermal in a 92:8 ratio, while beetles of three other *Trogoderma* spp. respond to the (*R*)-(−)-enantiomers of either (*E*)- or (*Z*)-trogodermal. Ratios of both geometrical and optical isomers of brevicomin determine response and pheromonal specificity of sympatric *Dryocoetes confusus* (Swaine) and *D. affaber* (Mannerheim) (Camacho et al., 1993).

Rhynchophorus palm weevils produce single isomers of chiral alcohols as aggregation pheromones (Rochat et al., 1991, 1993a,b; Oehlschlager et al., 1992, 1995; Gries et al., 1993; Hallett et al., 1993a,b; Zagatti et al., 1993; Weissling et al., 1994; Perez et al., 1994). 4-Methyl-5-nonanol (ferrugineol, **1**, Figure 1) is a male-produced aggregation pheromone of the two Asian palm weevils, *R. ferrugineus* (Oliv.) and *R. vulneratus* (Panz.) (Hallett et al., 1993a,b). 4-Methyl-5-nonanone (ferrugineone, **2**) is produced and elicits anten-

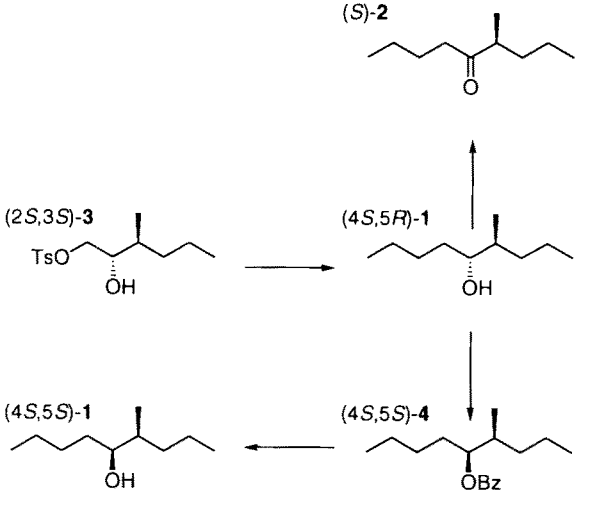

FIG. 1. Scheme for the synthesis of optical isomers of ferrugineol (**1**) and ferrugineone (**2**).

nal responses by both palm weevils, but seemed to have behavioral activity only in *R. ferrugineus* (Hallett et al. 1993a). Lack of pronounced differences in response to isomeric **1** and **2** suggested that pheromone chirality may impart species specificity of semiochemical communication. However, Perez et al. (1993) and Mori et al. (1993) demonstrated that both *R. ferrugineus* and *R. vulneratus* produce (*S,S*)-**1**, leading us to hypothesize that enantiospecific production of and response to **2** contributes to species isolation. We report the syntheses, as well as electrophysiological and behavioral activity of optical isomers of **1** and **2** in *R. ferrugineus* and *R. vulneratus*.

METHODS AND MATERIALS

Laboratory Analysis

Male and female *R. ferrugineus* and *R. vulneratus* were collected at Bojongkalong, West Java. Porapak Q-captured **1** and **2** from both weevils (Pierce et al., 1981; Hallett et al., 1993a) were subjected to gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975; Perez et al., 1994) (Hewlett Packard 5890A) and GC-mass spectrometry (MS) (Hewlett Packard 5985 B) on a fused silica, cyclodex-B-coated column (30 m × 0.25 mm ID, J&W Scientific, Folsom, California), which separates optical isomers of both **1** and **2** with baseline resolution. Chemical ionization (CI, isobutane) GC-MS analyses were conducted in both full-scan and selected-ion-monitoring mode (SIM). A full-scan mass spectrum of synthetic **1** and **2** was obtained to select diagnostic ions. For GC-MS CI-SIM, synthetic **1** and **2**, hexane and concentrated weevil-produced pheromone were injected in split mode and analyzed by scanning for diagnostic ions.

Instruments and General Procedures

Nuclear magnetic resonance (NMR) spectroscopy was conducted on a Bruker AMX-400 spectrometer at 400.13 and 100.62 MHz for ^{1}H and ^{13}C-NMR spectra, respectively. ^{1}H chemical shifts are reported relative to TMS and ^{13}C chemical shifts are referenced to $CDCl_3$. Gas chromatographic analyses were performed on Hewlett-Packard 5880A and 5890 instruments equipped with flame ionization detectors and fused silica, DB-1 coated columns (15 m × 0.25 mm ID; 0.25 mm film, J&W Scientific). Elemental analyses were performed using a Carbo Erba model-1106 Elemental Analyzer. Diethyl ether (Et_2O) and benzene were freshly distilled from sodium-benzophenone-ketyl and activated 4 Å molecular sieves, respectively. Chemicals obtained from commercial sources were used without further purification unless otherwise indicated. All moisture- and air-sensitive reactions were conducted under argon. Column chromatography

refers to flash chromatography using Silica Gel 60 (230–400 mesh, E. Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was conducted on aluminum-backed plates precoated with Merck Silica Gel 60F-254 as the adsorbent and visualized by treatment with an acidic solution of 1% $Ce(SO_4)_2$ and 1.5% molybdic acid followed by gentle heating.

The stereoisomers of **1** and **2** were synthesized according to a method modified from Perez et al. (1994). Oxidation of (4*S*,5*R*)-**1** and (4*R*,5*S*)-**1** were performed using the procedure of Brown et al. (1971).

(4S,5R)-5-Methyl-nonan-5-ol [(4S,5R)-1]. This compound was prepared by the alkylation of (2*S*,3*S*)-3-methyl-1-tosyloxyhexan-2-ol (**3**) according to the procedure of Perez et al. (1994). Enantiomer (4*S*,5*R*)-**1** was obtained as a colorless liqud. bp 75–78°C at 18 mm Hg; ^1H ($CDCl_3$): δ 0.88 (3 H, d, J = 8 Hz), 0.90 (6H, m) 1.34 (12H, m), 3.44 (1 H, m); ^{13}C ($CDCl_3$): δ 76.07, 38.55, 34.12, 33.09, 28.32, 22.78, 20.40, 15.24, 14.33, 14.04; Cl-MS *m/z* (relative intensity): 140 (100) (M^+-H_2O); Anal. calcd. for $C_{10}H_{22}O$: C, 75.87; H, 14.02; found: C, 76.10; H, 13.98 (4*R*,5*S*)-**1**: bp 80–83°C at 22 mm Hg; 2.62 g, 83% yield, 95% ee; Anal. Calcd. for $C_{10}H_{22}O$: C, 75.87; H, 14.02; found: 75.62; H, 13.97.

[(4S,5S)-4-Methyl-5-nonyl]benzoate [(4S,5S)-4]. Triphenylphosphine (7.87 g, 30 mmol), benzoic acid (3.66 g, 30 mmol), and (4*S*,5*R*)-**1** (2.39 g, 15 mmol) in 100 ml of dry benzene were stirred for 10 min at −10°C before diisopropyl azodicarboxylate (6.07 g, 5.9 ml, 30 mmol) (Aldrich Chemical Co., Milwaukee, Wisconsin) was added via syringe. After stirring overnight at room temperature, pentane was added, resulting in the formation of a white precipitate. The reaction mixture was filtered through a Florisil pad and concentrated in vacuo. Purification by column chromatography (9 : 1, pentane–ether as eluant), and by TLC using the same solvent system (R_f = 0.61) afforded (4*S*,5*S*)-**4** (2.46 g, 63% yield) as a pale yellow liquid: ^1H ($CDCl_3$): δ 0.90 (6H, m), 1.0 (3H, d, J = 8.9 Hz), (1.20 (2H, m), 1.36 (5H, m), 1.51 (1H, m), 1.60 (1H, m), 1,70 (1H, m), 1.98 (1H, m), 5.10 (1H, m), 7.48 (2H, ddd, J = 7.5, 1 Hz), 7.55 (1H, ddd, J = 7.5, 1Hz), 7.56 (2H, dd, J = 7.5 1Hz); ^{13}C ($CDCl_3$): δ 166.33, 132.59, 130.97, 129.54, 128.27, 125.48, 77.94, 36.19, 35.41, 31.23, 27.95, 22.62, 20.31, 14.41, 14.02, 13.92; Cl-MS *m/z* (relative intensity): 262 (2), 140 (M^+-C_6H_5-CO, 100)]; Anal. calcd. for $C_{17}H_{26}O_2$: C, 77.81; H, 9.99; found: C, 77.71; H, 10.17. (4*R*,5*R*)-**4**: 3.06 g, 73% yield; Anal. calcd. for $C_{17}H_{26}O_2$: C, 77.81; H, 9.99; found: C, 77.76; H, 9.90.

(4S,5S)-4-Methyl-5-nonanol [(4S,5S)-1]. Saponification of (4*S*,5*S*)-**4** (Perez et al., 1994) yielded (4*S*,5*S*)-**1** (1.20 g, 81% yield, 98% ee) as a colorless liquid: bp 72–74°C at 17 mm Hg; $[\alpha]_D^{20}$ = −25.5° (c = 1.765, Et_2O); ^1H ($CDCl_3$): δ 0.86 (2H, d, J = 8Hz), 0.90 (6H, m), 1.18 (2H, m), 1.34 (11H, m), 3.48 (1H, m); ^{13}C ($CDCl_3$): δ 75.19, 37.91, 35.64, 34.19, 28.45, 22.75, 20.43, 14.27, 14.00, 13.50; Anal. calcd. for $C_{10}H_{22}O$: C, 75.87; H, 14.02;

found: C, 76.01; H, 13.90. (4*R*,5*R*)-**1**: bp 65–68°C at 10 mm Hg; $[\alpha]_D^{20}$ = +26.6° (c = 1.895, Et_2O); 1.59 g, 85% yield, 98% ee; Anal. calcd. for $C_{10}H_{22}O$: C, 75.87; H, 14.02; found: C, 76.00; H, 13.99.

(S)-4-Methyl-5-nonanone [(S)-2)]. Oxidation of (4*S*,5*S*)-**1** (Brown et al., 1971) yielded (*S*)-**2,** bp 48–50°C at 20 mm Hg; $[\alpha]_D^{20}$ = +16.3° (c = 2.220, Et_2O); (2.34 g, 83% yield, 95% ee). IR (neat) 2959, 2874, 1713, 1460, 1409, 1378, 1124, 1046, 989 cm^{-1}; ^1H ($CDCl_3$): δ 0.90 (6H, m), 1.04 (3H, d, *J* = 8Hz), 1.29 (5H, m), 1.55 (2H, m), 1.60 (1H, m), 2.42 (2H, ddd, *J* = 8, 4Hz), 2.52 (1H, m); ^{13}C ($CDCl_3$): δ 214.94, 46.12, 40.84, 35.23, 25.86, 22.32, 20.49, 16.32, 14.09, 14.02; Anal. calcd. for $C_{10}H_{20}O$: C, 76.85; H, 12.91; found: C, 76.75; H, 12.86. (*R*)-**2:** bp 42–45°C at 10 mm Hg; $[\alpha]_D^{20}$ = −13.4° (c = 2.390, Et_2O); 2.40 g, 95% yield, 95% ee; Anal. calcd. for $C_{10}H_{22}O$: C, 76.85; H, 12.91; found: C, 77.03; H, 13.11.

Optical purities of (4*S*,5*R*)-**1** (96%), (4*R*,5*S*)-**1** (95%), (4*S*,5*S*)-**1** (98%), (4*R*,5*R*)-**1** (98%), (4*S*)-**2** (95%), and (4*R*)-**2** (95%) were determined by GC analyses on a Cyclodex-B column. Racemic **1** and **2** were synthesized as previously described (Hallett et al., 1993a).

Field Experiments

Response of *R. ferrugineus* and *R. vulneratus* to chiral isomers of **1** and **2** were investigated in coconut plantations at the Coconut Research Station, Pakuwon, West Java (August–December 1993, July and November–December 1994). Bucket traps (Oehlschlager et al., 1993a) were attached at 2 m height or at the base of coconut palms in randomized complete blocks. There are no significant differences in trap effectiveness for these two trap placements (Oehlschlager et al., 1993b; R.H. Hallett, unpublished data). Minimum intertrap and interblock distances were 16 and 45 m, respectively. All traps contained 1 kg of freshly cut coconut stem (approx. 5 × 5 × 20 cm) treated with a 0.3% a.i. solution of Basudin 60 EC (diazinon, Ciba-Geigy) to retain captured weevils.

Compounds **1** and **2** were released from 1-mm-ID capillary tubes (0.1 mg/day at 25°C) opened 1 cm above the liquid meniscus and placed inside 400 μl Eppendorf tubes. Eppendorf tubes had four 3-mm-diameter holes 1 cm below the top and were suspended 3–4 cm below the trap lid.

An experiment with four treatments and 20 replicates (August 16–30, 1993) tested attraction of both *R. ferrugineus* and *R. vulneratus* to coconut tissue alone or in combination with stereoisomeric **1** (0.4 mg/day at 25°C), (*S*,*S*)- or (*R*,*R*)-**1** (0.1 mg/day at 25°C). A second experiment with four treatments and 20 replicates (November 29–December 4, and December 7–15, 1993) tested attraction of both *R. ferrugineus* and *R. vulneratus* to coconut stem alone or in combination with racemic (0.4 mg/day at 25°C), (*S*)-, or (*R*)-**2** (0.2 mg/day at 25°C). A final experiment (four treatments, 19 replicates) (July 9–21, and

November 21–December 6, 1994) tested attraction of both *R. ferrugineus* and *R. vulneratus* to (*S*,*S*)-**1** (1 mg/day at 25°C) plus coconut stem alone or in combination with either racemic (0.2 mg/day at 25°C), (*S*)- or (*R*)-**2** (0.1 mg/day at 25°C), approximating the natural 10:1 ratio (Hallett et al., 1993a).

Statistical Analysis

Despite transformation by $\log(x + 1)$, data for all field experiments were not normally distributed and were therefore subjected to analysis by χ^2 tests ($\alpha = 0.05$) for species and treatment differences (Zar, 1984).

RESULTS AND DISCUSSION

Stereoisomers of **1** were produced according to rules used for preparation of structurally related pheromones of *R. phoenicis* and *R. cruentatus* (Figure 1) (Perez et al., 1994). Sharpless epoxidation of (*E*)-2-hexen-1-ol proceeded with high enantioselectivity unlike that of the *Z* isomer (Perez et al., 1994). Enantiomeric epoxides were stereo- and regiospecifically cleaved with trimethylaluminum and tosylated to produce (2*S*,3*S*)-3-methyl-1-tosyloxyhexan-2-ol [(2*S*,3*S*)-**3**] and its antipode. These derivatives were alkylated via cuprate (Grignard) displacement of the tosylate (Fouquet and Schlosser, 1974) to produce (4*S*,5*R*)-**1** (*anti*) and its antipode. The hydroxyl-bearing carbons of both stereoisomers were inverted through Mitsunobu chemistry to produce (4*S*,5*S*)-**1** and its antipode in high optical purity (95–98% ee).

Ketone (*R*)-**2** has previously been prepared in excellent optical purity (98% ee) using 1-amino-2-methoxymethylpyrrolidine as a chiral auxiliary (Enders et al., 1984). Although both enantiomers of the chiral auxiliary are available, this method requires stoichiometric amounts of these expensive reagents. Since the stereoisomers of **1** were obtained with very high enantiomeric excess, we investigated oxidation to the corresponding ketone (Figure 1). Jones' oxidation of alcohols possessing chiral α-carbons proceeds without loss of optical activity if conducted in a two-phase system ($Et_2O:H_2O$) (Brown et al., 1971). Applying Brown's procedure, (4*S*,5*R*)-**1** and its antipode yielded (*S*)-**2** and (*R*)-**2**, respectively, in high chemical yield and optical purity. Synthesis of ketones (*S*)-**2** and (*R*)-**2** in 30–35% overall yield (95% ee) based on 2-hexen-1-ol is comparable to the 44% overall yield (Enders et al., 1984) obtained in the previous four-step synthesis of (*S*)-**2**.

Gas chromatographic analysis of weevil-produced and synthetic (stereoisomeric) **1** on a SP-1000 column revealed that both *R. ferrugineus* and *R. vulneratus* produced the *syn* diastereoisomer (Perez et al., 1993, 1994). Further GC-EAD and GC-MS-SIM analyses on a Cyclodex-B column disclosed that

both palm weevils produce and antennally respond only to (4*S*,5*S*)-**1** (Figures 2 and 3). Production of (4*S*,5*S*)-**1** as an aggregation pheromone for these two species confirms previous results (Perez et al., 1993; Mori et al., 1993); (4*S*,5*S*)-**1** has also been reported as an aggregation pheromone for *R. bilineatus* (Montr.) in Papua New Guinea (Oehlschlager et al., 1995).

In field experiments, (±)-**1** or (4*S*,5*S*)-**1**, but not (4*R*,5*R*)-**1**, significantly enhanced attraction of both *R. ferrugineus* and *R. vulneratus* to traps baited with coconut stem tissue (Figure 4). No significant differences in attraction were found between the two species ($\chi^2 = 0.467$, $df = 3$, $P > 0.90$). As reported

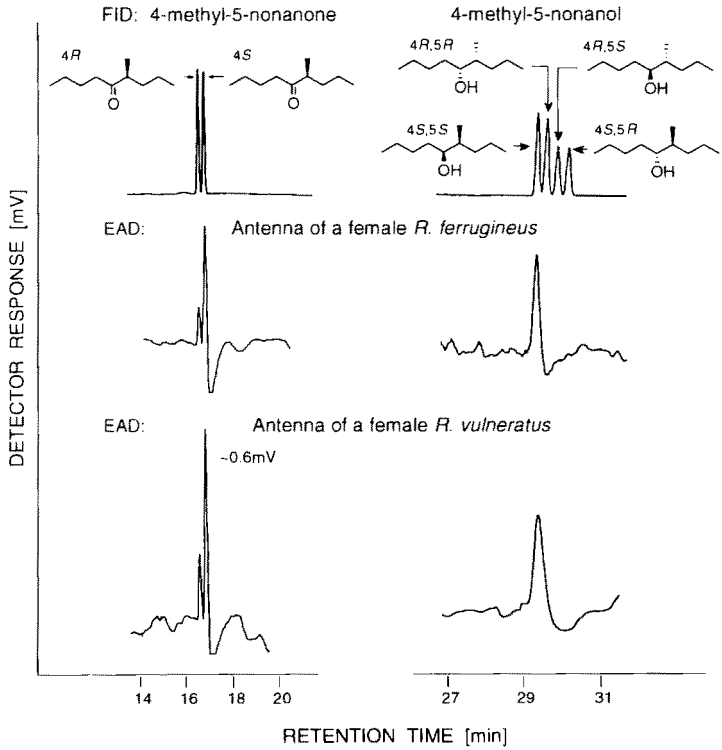

FIG. 2. Representative GC-EAD recordings of female *R. Ferrugineus* and *R. vulneratus* antenna responding to stereoisomers of 4-methyl-5-nonanone (**2**) and 4-methyl-5-nonanol (**1**) (Hewlett Packard 5890A; Cyclodex-B column; 100°C isothermal; split injector; linear flow velocity of carrier gas: 35 cm sec^{1}; injector temperature: 220°C). Different weevil antennae and chart speeds of 0.5 and 1.0 cm/min were used for the analyses of **2** and **1**, respectively.

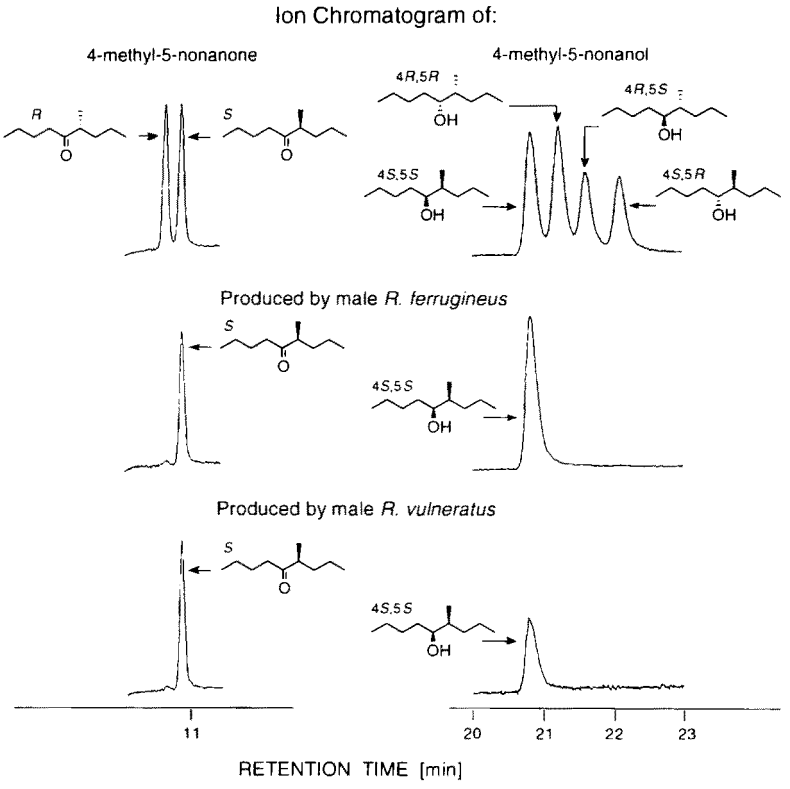

FIG. 3. Selected ion *m/z* 157 and *m/z* 141 chromatograms (Hewlett Packard 5985B GC-MS) of stereoisomeric and weevil-produced 4-methyl-5-nonanone (**2**) and 4-methyl-5-nonanol (**1**). *m/z* 157 and *m/z* 141 were, respectively, the parent ions of the full-scan mass spectra in CI mode of **2** [$(M^+ + H)$ 157] and **1** [$(M^+ + H)$ 159, $(M^+ - H_2O)$ 141] (column and chromatographic conditions as in Figure 2). Relative abundance of **2** and **1** in these ion chromatograms do not represent natural ratios.

in other Rhynchophorinae, nonnatural stereoisomers of the aggregation pheromone neither enhanced nor reduced attraction to the natural isomer (Oehlschlager et al., 1992, 1995; Perez et al., 1993, 1994).

Because (4*S*,5*S*)-**1** is an aggregation pheromone in both *R. ferrugineus* and *R. vulneratus*, we hypothesized that enantiospecific production of and response to **2** may impart species specificity of pheromone communication. However, GC-EAD and GC- MS-SIM analyses on a Cyclodex-B column of weevil-produced **2**, synthetic (±)-**2**, and each of the two enantiomers indicated that both

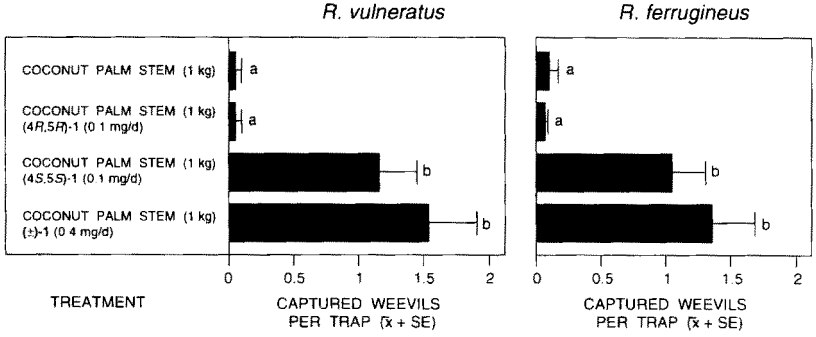

FIG. 4. Mean captures of *R. ferrugineus* (χ^2 = 41.157, *df* = 3, $P < 0.01$) and *R. vulneratus* (χ^2 = 36.987, *df* = 3, $P < 0.01$) in bucket traps baited with stereoisomers of 4-methyl-5-nonanol (**1**) and coconut stem tissue (Coconut Research Station, Pakuwon, West Java) August 16–30 1993; $N = 20$). Means followed by the same letter are not significantly different (χ^2 test, $\alpha < 0.05$).

R. vulneratus and *R. ferrugineus* produce and antennally respond more strongly to (*S*)-**2** than to (*R*)-**2** (Figures 2 and 3). Field experiments at Balitka, West Java, using coconut stem alone or in combination with (±)-**2**, (*S*)-**2**, or (*R*)-**2** (data not shown), indicated no significant differences between treatments for *R. ferrugineus* (χ^2 = 3.60, *df* = 3, $P > 0.25$) and *R. vulneratus* (χ^2 = 4.259, *df* = 3, $P > 0.20$) and no significant differences in attraction between the two species (χ^2 = 0.154, *df* = 3, $P > 0.95$). Furthermore, comparison of capture rates of traps baited with coconut stem tissue and (4*S*,5*S*)-**1** plus either (±)-**2**, (*S*)-**2**, or (*R*)-**2** at the natural 10:1 ratio of **1**:**2** did not indicate any behavioral activity of (±)-**2** or its enantiomers (Figure 5). No significant differences were found between the two species (χ^2 = 1.699, *df* = 3, $P > 0.50$) or between the two time periods (*R. ferrugineus:* χ^2 = 6.350, *df* = 3, $P > 0.05$; *R vulneratus:* χ^2 = 3.206, *df* = 3, $P > 0.25$) in which the experiment was conducted.

Although **2** is antennally active in both species, the result of four experiments did not disclose behavioral activity for either species in Indonesia or for *R. ferrugineus* in the United Arab Emirates (Figure 5) (Hallett et al., 1993a,b). Furthermore, in our field experiment in Java, **2** did not have significant behavioral activity in either weevil and did not impart specificity of pheromone communication. Throughout their wide geographic distribution, populations of *R. ferrugineus* and/or *R. vulneratus* may exist that respond to **2**.

Although *R. ferrugineus* and *R. vulneratus* are distinguished primarily by differences in dorsal coloration (Wattanapongsiri, 1966), there are color inter-

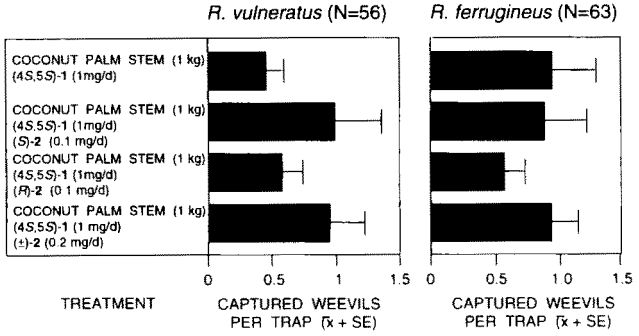

FIG. 5. Mean captures of *R. ferrugineus* and *R. vulneratus* in bucket traps baited with coconut stem tissue, (4*S*,5*S*)-4-methyl-5-nonanol (**1**), and isomers of 4-methyl-5-nonanone (**2**) (Coconut Research Station, Pakuwon, West Java, July 9–21 and November 21–December 6, 1994; $N = 19$. Data pooled for two time periods.). Means are not significantly different (χ^2 test, $\alpha = 0.05$).

morphs between the two species (Hallett et al., 1993a). Moreover, they are sympatric with similar food preferences (Wattanapongsiri, 1966), and in captivity exhibit interspecific copulation. In the scolytid genus *Ips*, closely related species are cross-attractive, but unlike *R. ferrugineus* and *R. vulneratus* maintain reproductive isolation through allopatric or parapatric distributions (Lanier and Wood, 1975). Failing the demonstration of other pre- or postzygotic reproductive isolating mechanisms, such as nonviability of progeny, *R. ferrugineus* and *R. vulneratus* should be synonymized.

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CORRELATION OF 1-OCTEN-3-ONE WITH ANTIXENOTIC RESISTANCE IN SUBTERRANEAN CLOVER COTYLEDONS TO RED-LEGGED EARTH MITE, *Halotydeus destructor* (ACARINA: PENTHALEIDAE)

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Abstract—Artificially damaged cotyledons of subterranean clover (*Trifolium subterraneum* L.) released several volatile metabolites, including 1-octen-3-one, arising from lipid peroxidation. The amount of 1-octen-3-one produced was negatively correlated with feeding damage caused by the red-legged earth mite (*Halotydeus destructor*) in nine out of 10 resistant and susceptible *T. subterraneum* varieties tested. The EC_{50} of this compound in deterring mites from feeding in a membrane bioassay was 50 ppm. Cotyledon toughness was also involved in resistance. The resistant variety, S3615D, which has the lowest toughness value among the resistant varieties, produced the highest amount of 1-octen-3-one recovered from the headspace in 1 hr. Artificially damaged cotyledons of both susceptible Dalkeith and resistant DGI007, growing in shade, showed lower toughness, but had enhanced production of C_8 volatile compounds and were avoided by mites during a 3-hr feeding test. When both 1-octen-3-one content and cotyledon toughness value were taken as cofactors in resistance, the resultant multiplication value yielded a more significantly negative correlation with mite feeding damage scores within the 10 varieties than either factor alone. We conclude that 1-octen-3-one has a role in resistance of subclover cotyledon to the mite.

Key Words—Subterranean clover, *Trifolium subterraneum*, cotyledons, red-legged earth mite, *Halotydeus destructor*, deterrence, 1-octen-3-one, volatiles, host resistance, antixenosis.

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INTRODUCTION

The red-legged earth mite, *Halotydeus destructor* (Tucker) (Acarina: Penthaleidae) causes an estimated annual loss of $200 million in production from pastures in southern Australia. Subterranean clover (subclover), *Trifolium subterraneum* L., suffers serious damage from feeding by the mites, especially at the seedling stage. As a consequence, cotyledons and even whole seedlings can be destroyed by mites. Screening for resistant varieties at the seedling stage has therefore become a priority in the control of this pest and a number of resistant varieties have been selected (Gillespie, 1994).

Cotyledons of the resistant varieties show antixenotic properties, with less mites feeding on them and being less damaged than susceptible varieties (Jiang and Ridsdill-Smith, unpublished data). Physical toughness of the cotyledons, regarded as a possible defense against the mite, is negatively correlated with mite feeding damage in at least 16 varieties of subclover (Jiang and Ridsdill-Smith, unpublished data). An exception is the resistant variety S3615D, in which the toughness value is as low as susceptible varieties, suggesting the likelihood that other resistance mechanisms are involved.

Cotyledons of the resistant varieties are not completely immune to mite feeding (Gillespie, 1993; Ridsdill-Smith and Gillespie, 1993). Behavioral observations reveal that mites visit cotyledons of resistant varieties initially in choice tests and some feeding occurs on such cotyledons in nonchoice tests. Silver patches, caused by feeding damage, are visible on cotyledons of resistant as well as susceptible varieties within several minutes (unpublished), although they are limited to small areas for the former varieties. Electron microscopy studies have shown that feeding damage is largely a result of a mechanical process, with mite chelicerae making penetration holes in epidermal cells (Ridsdill-Smith et al., 1996). These observations led us to hypothesize that "antifeedant" properties are not detected until the mite penetrates and damages the cotyledons. Since a mite sucks the content from single cells and penetrates cells quickly, any plant response to cell damage would need to be produced rapidly to have an effect on a feeding mite. The release of volatile compounds can occur within seconds following damage to plant tissue, and such a process could play a significant role in determining resistance. It is possible to investigate the volatile compounds with artificially damaged cotyledons, since Dicke and Sabelis (1992) have demonstrated that artificially damaged Lima bean leaves produce virtually all the same volatile compounds induced by spider mite feeding damage.

We have shown that volatiles from artificially damaged cotyledons are strongly deterrent and/or repellent to the mite at certain concentrations when tested in a membrane sachet bioassay (Jiang et al., unpublished data). The volatile compounds collected from a resistant variety are more deterrent than those from a susceptible variety (Jiang et al., unpublished data). In this paper,

we present evidence that the volatile compound, 1-octen-3-one, was involved in resistance. Bioassay results indicated that the compound was deterrent to the mite in a concentration-dependent manner. Furthermore, in an experiment designed to find effects on mite feeding of cotyledons growing in the shade, it was found, unexpectedly, that although the cotyledons had significantly lower toughness, the mites did not feed on shaded cotyledons and production of 1-octen-3-one was increased in shaded cotyledons.

METHODS AND MATERIALS

Plant Material. Seeds of four susceptible and six resistant subclover varieties were sown in pots (each 12 cm diameter) with standardized sand–loam soil as described by Ridsdill-Smith and Gillespie (1993). For each test, two susceptible and two resistant varieties were grown under the same glasshouse conditions. Seedlings were harvested and processed on the same day for collection of volatile compounds, 12 or 13 days after seed sowing. To study the effects of toughness and volatiles of cotyledons growing in the shade on feeding of mites, seedlings in five pots of each variety (Dalkeith, susceptible; DGI007, resistant) were covered with a cardboard box with four openings of 5 × 5 cm in the middle of the side walls, allowing in dim light to the plants. As controls, seedlings in five other pots were not covered. Cotyledons were used at 12 or 13 days after seed sowing.

Mites. Red-legged earth mites were collected from the field, near Perth, in winter. They were age-graded after collection and only young adult mites were used for bioassays. Before the bioassay, mites were starved for 2 hr in a humid vial.

Chemicals. 1-Octen-3-ol and 2-(*E*)-hexenal were purchased from Aldrich and Sigma Chemical Company, respectively. 1-Octen-3-one was prepared by oxidation of 1-octen-3-ol with pyridinium chlorochromate.

Headspace Collection of Volatile Compounds from Artificially Damaged Cotyledons. A stream of air filtered through granulated charcoal (about 50 g) was drawn into a separating funnel (8 cm diameter) that contained the plant tissues. The air carrying the volatile compounds was passed into a trap made of 50 mg powdered charcoal in a Pasteur pipet. The airflow was controlled by the water pump (about 500 ml/min). Trapping was generally conducted for 1 hr only, unless specified otherwise. For quantitative comparison among 10 varieties, 2.4 g of cotyledon tissues of each variety were used. To determine the 1-octen-3-one content after collection for different times (1 and 6 hr), 7.8 g of cotyledon tissues were used for each variety. To test cotyledons grown in the shade, 3.5 g of tissues were used. Detached cotyledons were frozen in liquid nitrogen and ground (damaged) in a mortar. The powdered tissues were trans-

ferred, with a filter paper, into a round funnel (8 cm diameter), in which another filter paper (Whatman No. 1), cut netlike, was set at the bottom to hold the powder. The tissue was deposited on the walls of the funnel by turning it around. By this time, the tissue had thawed and collection of the volatile compounds was started. To compare different varieties, 5 or 10 μl of hexanol was added as an internal standard. The charcoal traps were stored at −20°C.

Gas Chromatography (GC). The volatile compounds were desorbed with 1 ml of dichloromethane. The compounds collected were analyzed by GC, using a Hewlett-Packard 5790A instrument equipped with a fused silica BP 1 column (0.25-μm film thickness, 0.22 mm ID × 25 m) and an FID detector with hydrogen as the carrier gas (1.5 ml/min). Temperature was programmed from 40°C for 5 min and then to 290°C at 20°C/min. For each sample, at least two chromatograms were recorded.

Gas Chromatography–Mass Spectrometry (GC-MS). Volatile compounds were analyzed using a Hewlett-Packard 5986 GC-MS (EI, 70 eV). The compounds were identified by comparison of their mass spectra and retention time, singly and in admixture, with those of authentic compounds.

Air Oxidation of 1-Octen-3-ol. 1-Octen-3-ol (1 μl; 98% purity) was treated under the same conditions as described for damaged cotyledon tissues. In another test, 1 ml of 0.05% (v/v) aqueous 1-octen-3-ol solution was added onto the frozen powders of ground cotyledon tissues of a resistant variety (DGI007) and the volatiles were collected as for other treatments.

Membrane Feeding Test. The bioassay of the volatile compounds was conducted by using membrane sachets as described in Jiang et al. (unpublished data). Briefly, compounds were added to pure Tween 80. A solution of glucose in distilled water was added to achieve a final concentration of 5% Tween 80 and 1% glucose. To measure the EC_{50} for deterrence, a range of concentrations of a compound were tested. The aqueous solution used as feeding medium was sandwiched between two layers of parafilm membranes stretched over a plastic ring (2 cm diameter). In the two-choice tests, a sachet with the test compound was a treatment, whereas the other contained only Tween 80 and glucose and served as a control. Two membrane sachets were set into the surface of a sand–loam soil close to each other in a tissue culture cup (5.7 cm diameter). Twenty mites were released onto the soil, and mite numbers were counted every 20 min for the first hour and then every 30 min afterwards for a further 2 hr. There were 10 replicates for each treatment. The averages from seven observations for each replicate were used to calculate the mean and standard errors, which were compared with a t test for statistical significance in a choice test. Mite counts provide a relative estimate of feeding (Jiang and Ridsdill-Smith, unpublished data) based on the formula [(numbers of mites on control − numbers of mites on treatment)/both mite numbers added)] × 100. Mite selection between the treatment and control membranes was expressed as deterrence of the test

compound (indicated by a positive value) or as preference for the test compound (indicated by a negative value).

Scores of Mite Feeding Damage to Cotyledons over Two Weeks. Seedlings in pots with multivariety choices were exposed to mites for two weeks (Gillespie, 1993). Mite feeding damage to seedlings was scored for each variety from more than two experiments with a 1–10 rating system (1 = 10% feeding damage and 100% = death of seedlings). Damage scores collected by Gillespie from different varieties were used in this study to calculate regression between the resistance and amounts of volatile compounds.

Cotyledon Choice Feeding Test. The choice feeding methods are described in Jiang and Ridsdill-Smith (unpublished data). The cotyledons were cut and two were placed close to each other on the sand–loam in a small Petri dish (5 dm diameter). Cotyledons from the same or a different variety (Dalkeith, susceptible; and DGI007, resistant) were compared for effects of shaded cotyledons on mites. Numbers of mites feeding on cotyledons were counted every 20 min in the first hour and then every 30 min afterwards for 2 hrs. There were 10 replicates for each choice test. Numbers of mites counted from the seven observations during 3 hr were averaged and used to calculate mean, standard error, and statistical significance (t test). Mite preference for a cotyledon was calculated with the above formula.

Toughness Test of Cotyledons. Toughness of cotyledons from varieties (Dalkeith, susceptible; and DGI007, resistant) growing in shade or light was measured with a pipet-balance penetrometer (Jiang and Ridsdill-Smith, unpublished data). Four penetrations were made for each cotyledon, on both sides of the main vein away from the base, with 10 replicate cotyledons for each treatment.

Combined Effects of Toughness and Volatiles. Based on the results obtained in this work for 1-octen-3-one and the results collected by Jiang and Ridsdill-Smith (unpublished data) for toughness, it was assumed that both 1-octen-3-one and toughness contributed to the resistance in cotyledons. It is unknown whether both factors are of equal importance in resistance, but values of both for a variety were multiplied to produce an index. Regressions were made between the index and damage scores for the different varieties.

RESULTS

Identification of Compounds Present in Headspace Collection. No qualitative difference in the volatile compounds produced was found among the varieties examined (Figure 1). Of the five major compounds, three, 2-(E)-hexenal, 1-octen-3-one, and 1-octen-3-ol, have been identified. Comparison of their mass spectrometric and chromatographic properties with those of authentic samples

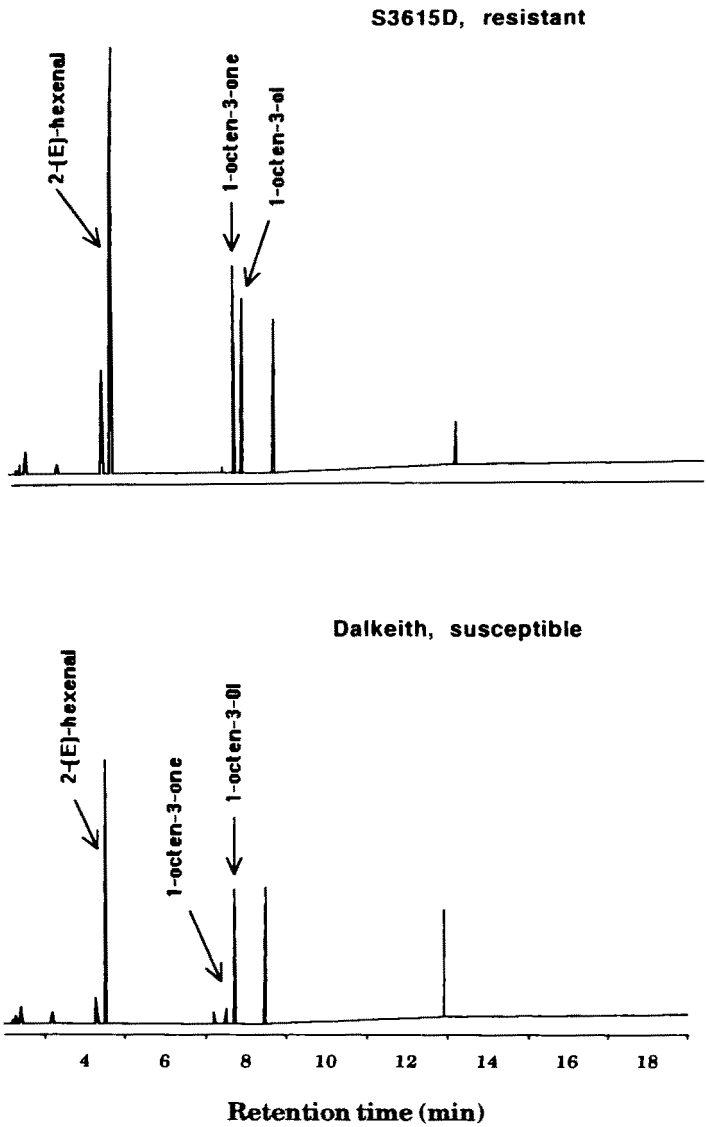

FIG. 1. Gas chromatograms of headspace volatile compounds collected from damaged cotyledons of subclover. Varieties shown here were S3615D (resistant) and Dalkeith (susceptible). Positions of 2-(*E*)-hexenal, 1-octen-3-ol, and 1-octen-3-one are indicated. Some volatile compounds are unidentified.

established their identity. Not more than 0.5% 1-octen-3-one was detected from the headspaces of either 1-octen-3-ol or combined 1-octen-3-ol and ground DGI007 cotyledon tissues, indicating negligible air oxidation of this metabolite.

Quantitation of Volatile Compounds and Correlation with Mite Feeding Damage. The relative amounts of the three compounds collected from 10 varieties over a 1-hr period are presented in Table 1. There was variation of the compounds between varieties, but only 1-octen-3-one was consistently higher in resistant than in susceptible varieties, with one exception (82S47.16.2). There is a significant negative correlation ($R^2 = 0.713$) between the levels of 1-octen-3-one produced and scores of mite feeding damage within a two-week testing period.

Collection of 1-octen-3-one over 6 hr yielded a higher amount than in 1 hr in all three varieties (Figure 2). The estimated amount of 1-octen-3-one collected within 1 hr from the damaged cotyledons was about 5 ppm in the susceptible varieties, whereas in resistant varieties it was about 15 ppm (19 ppm in S3615D). The amount of 1-octen-3-ol collected was generally higher than that of 1-octen-3-one and varied from 2 ppm (variety 82S47.16.2) to 28 ppm (variety S3617D). 2-(*E*)-Hexenal also varied from 11 ppm (variety Dalkeith) to 30 ppm (variety DGI007).

Antifeeding Activity of 1-Octen-3-one with Membrane Feeding Tests. At high concentrations, all three volatile compounds, 1-octen-3-one, 1-octen-3-ol,

TABLE 1. VOLATILE COMPOUNDS RELATIVE TO *n*-HEXANOL IN HEADSPACE OF ARTIFICIALLY DAMAGED COTYLEDONS (2.4 g) OF SUBCLOVER VARIETIES[a]

Varieties	Character	Compound (mean ± SE)		
		1-Octen-3-one	1-Octen-3-ol	2-(*E*)-Hexenal
Dalkeith	Susceptible	0.13 ± 0.001	0.44 ± 0.001	0.47 ± 0.026
Denmark	Susceptible	0.11 ± 0.001	0.21 ± 0.001	2.84 ± 0.010
Junee	Susceptible	0.11 ± 0.001	0.54 ± 0.002	2.53 ± 0.001
Seaton Park	Susceptible	0.12 ± 0.001	0.62 ± 0.001	0.55 ± 0.010
82S47.162	Resistant	0.15 ± 0.001	0.09 ± 0.001	2.07 ± 0.020
S3617D	Resistant	0.27 ± 0.003	1.18 ± 0.001	1.77 ± 0.005
S3623A	Resistant	0.39 ± 0.002	0.80 ± 0.005	1.41 ± 0.005
S3615H	Resistant	0.29 ± 0.001	0.31 ± 0.001	1.39 ± 0.010
DGI007	Resistant	0.39 ± 0.001	0.85 ± 0.003	4.95 ± 0.005
S3615D	Resistant	0.48 ± 0.005	0.65 ± 0.002	2.69 ± 0.005

[a]Collection of compounds was completed at 1 hr. The internal standard was 10 μl *n*-hexanol ($n = 2$).

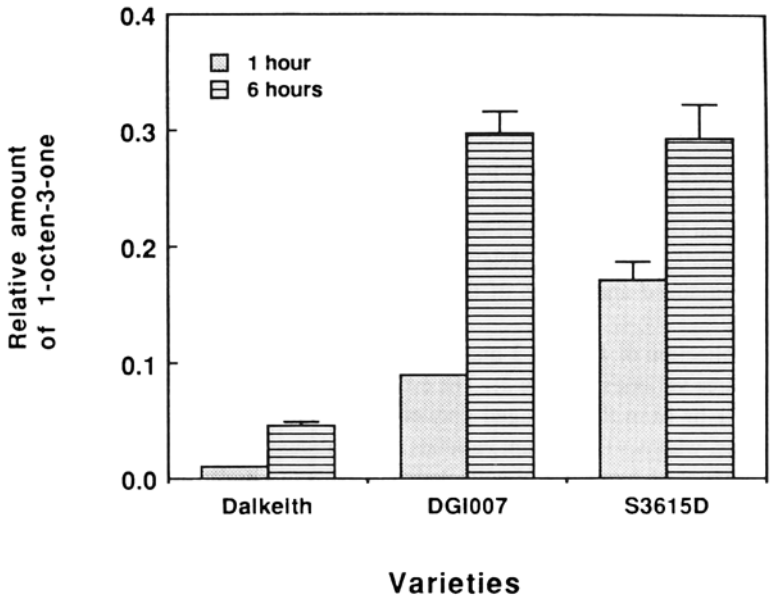

FIG. 2. Relative amounts of 1-octen-3-one collected for 1 and 6 hr from the headspace of damaged cotyledons of three subclover varieties, Dalkeith (susceptible), DGI007 (resistant), and S3615D (resistant).

and 2-(*E*)-hexenal, exhibited antifeeding activity towards mites in the membrane feeding test. This is illustrated for 1-octen-3-one (Figure 3). The EC_{50} for deterrent activity was 50 ppm for 1-octen-3-one, 55 ppm for 2-(*E*)-hexenal, and 285 ppm for 1-octen-3-ol. For 1-octen-3-one and 2-(*E*)-hexenal, all mites were repelled from feeding on both treatment and control membrane sachets with concentrations of 1000 ppm and higher. At low concentration (about 1 ppm), 1-octen-3-one and 2-(*E*)-hexenal were attractive to the mites in the three hour test.

Correlation of 1-Octen-3-one Content and Toughness in Relation to Mite Feeding Damage. Most resistant varieties tested not only produce high amounts of 1-octen-3-one but also have high toughness values (Jiang and Ridsdill-Smith, unpublished data), although 1-octen-3-one is low for variety 82S47.16.2 and toughness for variety S3615D (Figure 4). Multiplication between 1-octen-3-one content and the toughness value for each variety yields an index that was negatively correlated with mite feeding damage scores. The R^2 value (0.873) obtained was higher than that (0.713) in the correlation between 1-octen-3-one

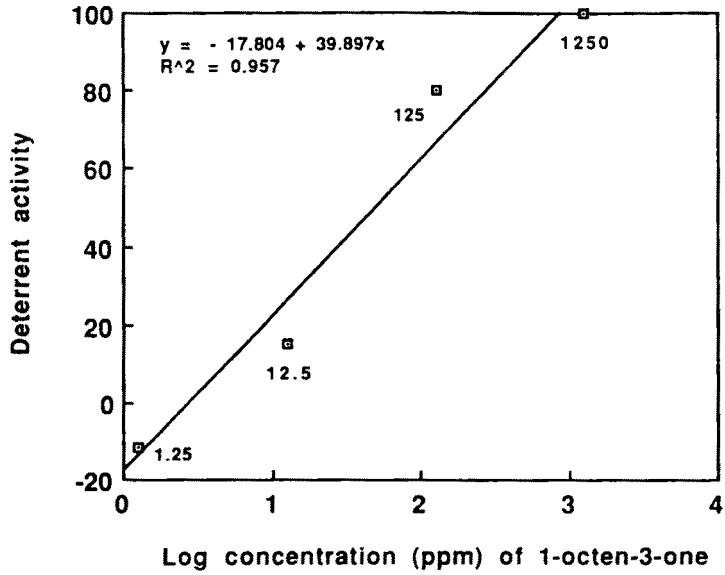

FIG. 3. Responses of the red-legged earth mite to 1-octen-3-one tested at four concentrations (1.25, 12.5, 125, and 1250 ppm) in a membrane bioassay. Note attractiveness of the compound at 1.25 ppm.

alone and damage score and that (0.752) in the correlation between toughness and damage score.

Shading Effect on Mite Feeding and Volatile Content. Cotyledons of both Dalkeith (susceptible) and DGI007 (resistant) grown under shading were smaller than nonshaded cotyledons, but they were green. The shaded cotyledons of both varieties were completely avoided by mites in choice feeding tests during 3 hr (Table 2). Nonshaded cotyledons of DGI007 were more acceptable than shaded Dalkeith cotyledons. Toughness test indicated that shaded cotyledons of both varieties had significantly lower toughness values ($P < 0.01$) than the nonshaded counterparts: with Dalkeith (susceptible), toughness was 59.1 $\pm$ 0.93 for nonshaded and 43.9 $\pm$ 0.46 for shaded cotyledons; with DGI007 (resistant), this was 81.8 $\pm$ 1.04 for nonshaded and 52.4 $\pm$ 1.15 for shaded cotyledons. However, analyses of volatile compounds showed that C_8 volatile compounds, especially 1-octen-3-one, increased by more than 50%, whereas 2-(*E*)-hexenal decreased 9% in both varieties with shading (Table 3).

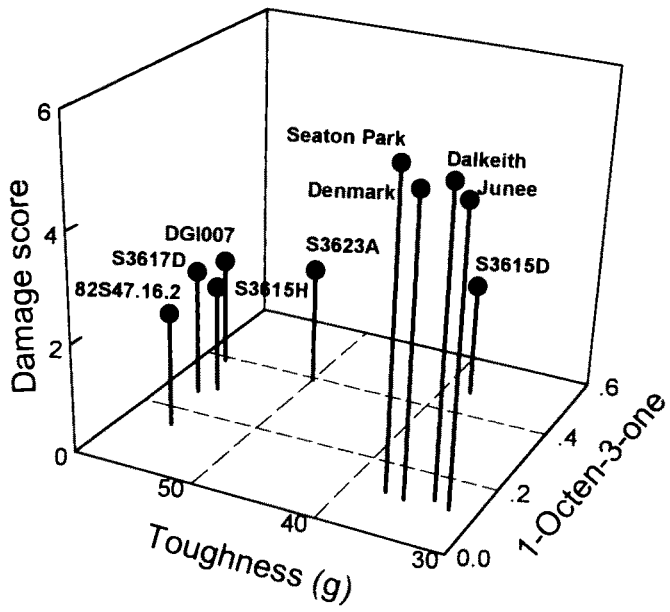

FIG. 4. Three-dimensional relationships between mite feeding damage scores, relative amount of 1-octen-3-one, and toughness value of the same 10 varieties of subclover. Note the four susceptible varieties are grouped as having both the lowest content of 1-octen-3-one and toughness values, whereas the resistant varieties are scattered around with either high 1-octen-3-one content or toughness values or both.

DISCUSSION

There is no visible difference in surface structure between subclover cotyledons of resistant and susceptible varieties. The cotyledons do not possess trichomes (Ridsdill-Smith et al., 1995), which are important chemical and physical barriers against spider mites in many other plant species. Furthermore, no antifeedant activity was found in nonvolatile extracts from resistant varieties, in comparison to susceptible varieties.

Higher amounts of 1-octen-3-one are produced in most resistant than in susceptible varieties, in particular the S-lines, which originate from Sicily, Italy. The significant correlation between 1-octen-3-one produced and resistance and its concentration-dependent deterrent activity strongly suggest that it could be responsible, at least in part, for the deterrent activity of the variety S3615D, which has low cotyledon toughness, as well as in other varieties with high cotyledon toughness such as DGI007. The concentration of 1-octen-3-one occur-

TABLE 2. MITE NUMBERS ON DETACHED COTYLEDONS GROWING UNDER SHADE (−) AND LIGHT (+) FROM TWO VARIETIES (DALKEITH, SUSCEPTIBLE; DGI007, RESISTANT) IN PAIRWISE CHOICE TESTS[a]

Choices	Mites ($N \pm$ SE)	Preference for the 1st choice	P level from t test
(+) Dalkeith-(− Dalkeith)	11.2 ± 0.38 − 1.4 ± 0.25	77.8	<0.001
(+) DGI007-(−) DGI007	7.5 ± 0.22 − 0.6 ± 0.27	85.2	<0.001
(−) Dalkeith-(−) DGI007	4.5 ± 0.19 − 1.7 ± 0.09	45.2	<0.001
(+) Dalkeith-(−) DGI007	12.4 ± 0.39 − 0.2 ± 0.05	96.8	<0.001
(−) Dalkeith-(+) DGI007	1.3 ± 0.26 − 8.2 ± 0.38	−72.6	<0.001

[a]There are highly significant differences between each choice ($P < 0.001$).

TABLE 3. SHADING EFFECTS ON RELATIVE AMOUNT OF VOLATILE COMPOUNDS IN HEADSPACE OF ARTIFICIALLY DAMAGED COTYLEDONS (3.5 g) OF SUBCLOVER VARIETIES DALKEITH (SUSCEPTIBLE) AND DGI007 (RESISTANT)[a]

Treatments	Compound (mean ± SE)		
	1-Octen-3-one	1-Octen-3-ol	2-(*E*)-Hexenal
Dalkeith (susceptible)			
Non-shaded	0.14 ± 0.001	0.70 ± 0.003	2.04 ± 0.001
Shaded	0.21 ± 0.002	1.16 ± 0.002	1.85 ± 0.005
DGI007 (resistant)			
Non-shaded	0.26 ± 0.005	1.11 ± 0.006	3.36 ± 0.010
Shaded	0.49 ± 0.005	1.17 ± 0.001	3.05 ± 0.010

[a]The difference between shaded and nonshaded cotyledons is significant within the same variety using the t test ($P < 0.05$). An internal standard of 5 μl n-hexanol was used in quantification ($N = 2$).

ring in the resistant variety S3615D (19 ppm, collected within 1 hr) is within the range of concentrations for which mite deterrence is observed. With the susceptible variety Dalkeith, we were able to demonstrate production of 2-(*E*)-hexenal, 1-octen-3-ol, and 1-octen-3-one from cotyledons damaged by red-legged earth mites feeding activity (unpublished). Thus, the volatile compounds pro-

duced by artificially damaged cotyledons are representative of those produced by cotyledons after mite feeding.

The two- to threefold increase in 1-octen-3-one collected with time suggests that the compound is produced in a time-dependent manner and/or that collection within 1 hr underestimates the amount of compounds. Because the mites readily distinguish between cotyledons of resistant and susceptible varieties within 1 hr, it is reasonable to correlate mite resistance with the compounds collected within 1 hr. Terpenoids do not appear to be present in detectable amounts. We have shown that this is not due to the trapping system employed, since terpenes from damaged *Eucalyptus* leaves were trapped effectively using the same technique (unpublished). Dicke and Sabelis (1992) also found no production of terpenes in artificially damaged Lima bean leaves.

The striking negative effect of shade-grown cotyledons on mite feeding behavior and on the relative proportions of the volatile compounds are consistent with the results above. Shade-grown cotyledons have much lower toughness than nonshaded ones, but mites apparently do not like gathering and feeding on them. Although other changes, such as morphology and content of sugar, in these cotyledons could also affect mite feeding behavior, production of C_8 compounds (1-octen-3-one and 1-octen-3-ol) in both shade-grown susceptible and resistant varieties increased considerably. This could explain, at least in part, the reduced acceptance of the cotyledons by mites. The DGI007 variety is highly resistant to the red-legged earth mite (Gillespie, personal communication), and it appears that both high toughness value and high 1-octen-3-one production contribute to this resistance. However, the experiments with shade-grown cotyledons tend to suggest that the role of 1-octen-3-one in resistance is more important than toughness.

2-(*E*)-Hexenal has been shown to arise from the unsaturated fatty acid, linolenic acid (Hatanaka, 1993; Vick and Zimmerman, 1987). This is present in greater amounts in green leaves, particularly in the membranes of chloroplasts, than in nongreen and dark-grown leaf tissues (Harwood, 1980). On the other hand, the C_8 volatile compounds originate from linoleic acid (Mau et al., 1994), which is produced in greater amounts in dark-grown leaves than in green leaves and more in white than in the green parts in *Chlorophytum* (Harwood, 1980).

The above observations further support the correlation between 1-octen-3-one and mite resistance and help to rationalize the lack of correlation between levels of 2-(*E*)-hexenal and mite resistance. We presume that 2-(*E*)-hexenal collected from artificially damaged cotyledons is produced by the green palisade and spongy mesophyll cells that are not the major feeding sites of the mite. However, C_8 volatile compounds are probably produced, for the most part, in the nongreen epidermal cells. If this is true, the C_8 compounds should have a

much higher local concentration than is revealed from headspace trapping experiments.

Hildebrand et al. (1986) have observed enhanced lipid peroxidation and lipoxygenase activity in soybean leaves infested with spider mites. The authors suggest that the metabolite hexenal played a role in the induced resistance of soybean to spider mite (Kasu et al., 1994). We are not in a position to judge whether or not hexenal could also be involved in resistance because our measurements were for the whole cotyledon tissue, rather than for those specific tissues subjected to feeding mites.

In conclusion, the results strongly suggest that wound-induced C_8 compound, 1-octen-3-one, plays a significant role in the deterrence of cotyledons of some resistant subclover varieties to the red-legged earth mites. The combined effects of the compound and physical toughness confer more resistance to a variety than either factor alone. In the future, we need to establish whether or not other chemical compounds, e.g., those specifically induced by mite feeding, are also involved in the antixenotic resistance of subclover cotyledons to the redlegged earth mite.

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ANAL GLAND SECRETION OF EUROPEAN MOLE: VOLATILE CONSTITUENTS AND SIGNIFICANCE IN TERRITORIAL MAINTENANCE

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Abstract—The chemical composition of the volatile components of the anal gland secretion of mature and immature moles of both sexes was examined by gas chromatography and mass spectrometry. The compounds in the secretion of adult males appear to vary little throughout the year and are similar to those from the adult female outside the breeding season. The secretions are dominated by C_5–C_{10} carboxylic acids. Female glands regress as they enter proestrus, accompanied by profound changes in the chemical composition of the secretion with early disappearance of the carboxylic acids. In juvenile moles, the compostion of the secretion changes as the animal matures, with carboxylic acids becoming dominant only as the animal reaches maturity. Anal gland secretion probably plays an important role in territorial maintenance.

Key Words—Moles, *Talpa europaea*, anal glands, scent marking, territoriality, semiochemical, proestrus, carboxylic acids.

INTRODUCTION

For most of the year the European mole (*Talpa europaea*) lives a solitary life in its own subterranean tunnel system and actively avoids contact with other members of its own species. The home ranges of females or of a male and a female may, however, overlap, and lengths of tunnel may be used in common. Moles in overlapping territories are aware of each other's existence and move-

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ments, and they will organize their daily routine so as to avoid each other (Stone and Gorman, 1985; Gorman and Stone, 1990). The only time of the year when moles purposefully meet is during the mating season, when male moles will migrate into female territories in order to mate (Godfrey and Crowcroft, 1960). Studies by Gorman and Stone (1990) have suggested that the mole's efficient territorial maintenance is controlled at least in part by chemical signals produced by the secretion of specialized glands in the inguinal region of both sexes. These glands have been termed "preputial glands" by some workers (Racey, 1978; Stone and Gorman, 1985; Gorman and Stone, 1989, 1990), but we have recently shown that they are in fact anal glands (Clevedon Brown et al., 1994).

We present results here that support the hypothesis that the secretion from the anal glands plays a central role in the territorial maintenance of the mole. Our evidence is based on studies on variations in glandular mass and the chemical composition of the volatile components of the glandular secretion throughout the year.

METHODS AND MATERIALS

Moles used for both the anatomical and chemical studies were caught in lethal traps in Cambridgeshire and adjacent parts of Norfolk by a professional mole catcher (DRE Pest Control, Swavesey, Cambridgeshire). The carcasses were frozen immediately on collection for transport to the laboratory, where they remained frozen at −20°C until dissection. Anal glands were separated from extraglandular material and weighed. The glandular secretion was released by crushing the gland, and the volatile components therein were trapped on an activated charcoal disc using a Gorb dynamic headspace apparatus as previously described (Buglass et al., 1990). The volatiles were eluted from the disc with 8 μl of dichloromethane containing dihexyl sulfide (115 ng/μl as internal standard), and 1 μl of the resulting solution injected into a Hewlett-Packard 5890 gas chromatograph equipped with a polydimethylsiloxane column (length, 15 m; ID, 0.25 mm) and connected to a Hewlett-Packard 5970 mass spectrometer detector. The temperature program was 35–240°C at a rate of 4°C/min with a final hold at 240°C for 45 min. Compounds were identified from their mass spectra and also, in most cases, by comparison of retention times with those of authentic compounds (see Table 1 below).

Male moles in breeding condition were distinguished from nonbreeding males by the great increase in size of the testes and epididymus. Proestrus in the female was characterized by the presence of the vaginal opening, the increase in length and breadth of the reproductive tract resulting from growth and thickening of its wall, and vascularization of the uterine cornua (Harrison Matthews, 1935). We observed an increase in length of the uterovaginal canal to around

40–45% of body length during proestrus, as opposed to a normal value of less than 20%, in line with the findings of Harrison Matthews (1935). As estrus itself lasts for only about 30 hr in the mole (Godfrey and Crowcroft, 1960), no attempt was made to distinguish between the proestrous and the estrous female.

RESULTS AND DISCUSSION

In East Anglia the mating season for moles occurs early in the year. Females approach estrus as early as January. The gestation period is four weeks and lactation follows for a further four weeks (Deanesly, 1966; Godfrey and Crowcroft, 1960; Harrison Matthews, 1935); hence by February or March, females are likely to be pregnant or to be lactating. Additionally, we have observed a low incidence of second proestrus and presumably estrus in September.

Preliminary results from our study of the changes in anal gland size during the year showed that the glands of males and females behave differently (Buglass et al., 1995). Male glands increase in weight during the breeding season, reaching a maximum value at the peak of the breeding season in February and March. This change is probably due to vascular development resulting from a surge in testosterone, which in the shrew serves to increase vascularity of sebaceous tissue (Pearson, 1946). The increase in size and weight of the gland is associated with a deepening of its color to dark red. In contrast, female glands undergo a reduction in size as the female enters estrus in January or February. In the majority of 14 females that were collected in January, the glands had regressed to such a degree that they were invisible to the naked eye. Female glands enlarge as the breeding period ends, but the average value drops sharply in May before climbing again during summer and fall. We explain this effect by the inclusion of juveniles in the catch from May onwards. The glands of juveniles are flaccid and less developed than those of the mature mole, and it is not until August or September that glands of moles born in that year become indistinguishable from those of mature animals. The results of an extension of this study, involving a greater number of moles, are shown in Figure 1. These results are in full agreement with our earlier findings.

Samples of anal gland secretion from a total of 130 moles were analyzed by gas chromatography and mass spectrometry. For adult moles, chromatograms were obtained from secretion collection in each month of the year. A typical chromatogram from a nonbreeding male, a breeding male, a juvenile male, a nonbreeding female, a proestrous female, a pregnant female, a lactating female, a female approaching second estrus, and a juvenile female are shown in Figure 2A–I, respectively. Compounds are identified in Table 1.

With the exception of the proestrous female, C_5–C_{10} carboxylic acids, both straight and branched chain, form a significant component of the secretions of

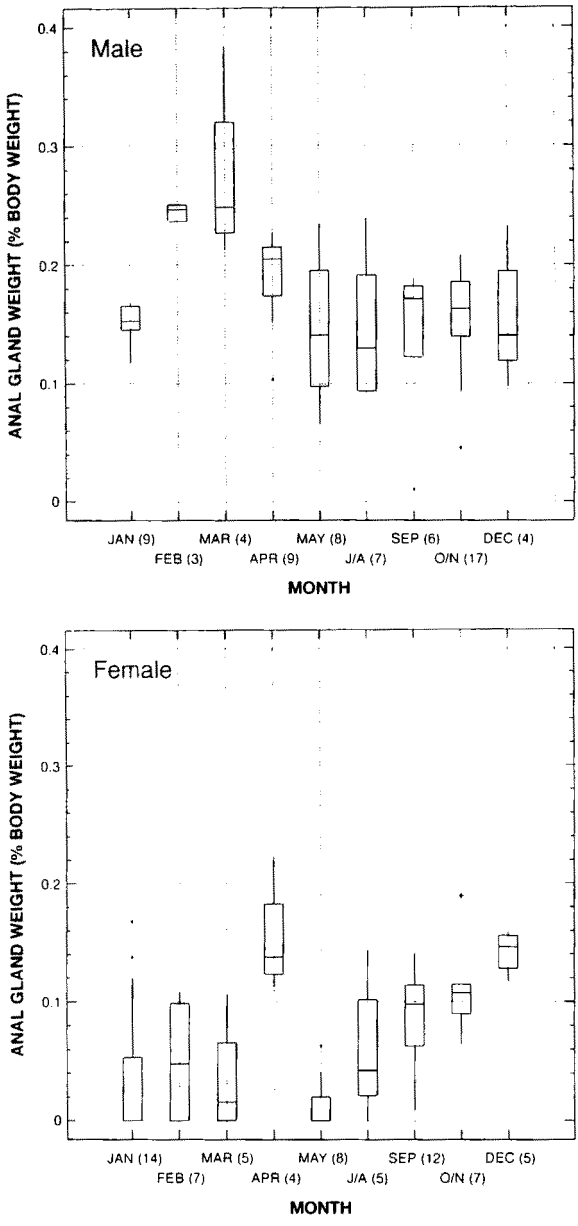

FIG. 1. Box and whisker plots showing the seasonal changes in the anal gland weight as a percentage of body weight for male and female moles. Numbers refer to number of moles in each sample.

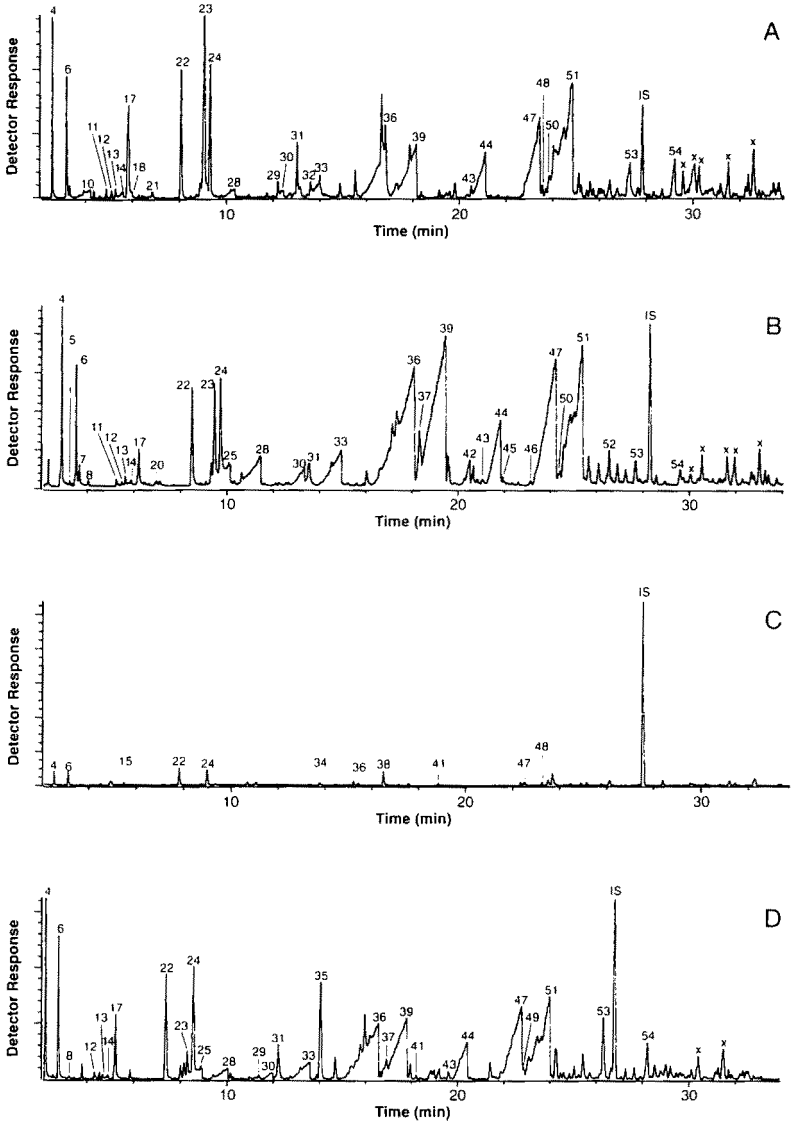

FIG. 2. Gas chromatograms of volatile compounds from the anal gland secretions of moles: (A) nonbreeding male (October); (B) breeding male (April); (C) juvenile male (May); (D) nonbreeding female (October); (E) proestrous female (January); (F) pregnant female (February); (G) lactating female (March); (H) female approaching second estrus (September); (I) juvenile female (May). x = impurity; IS = internal standard (dihexyl sulfide).

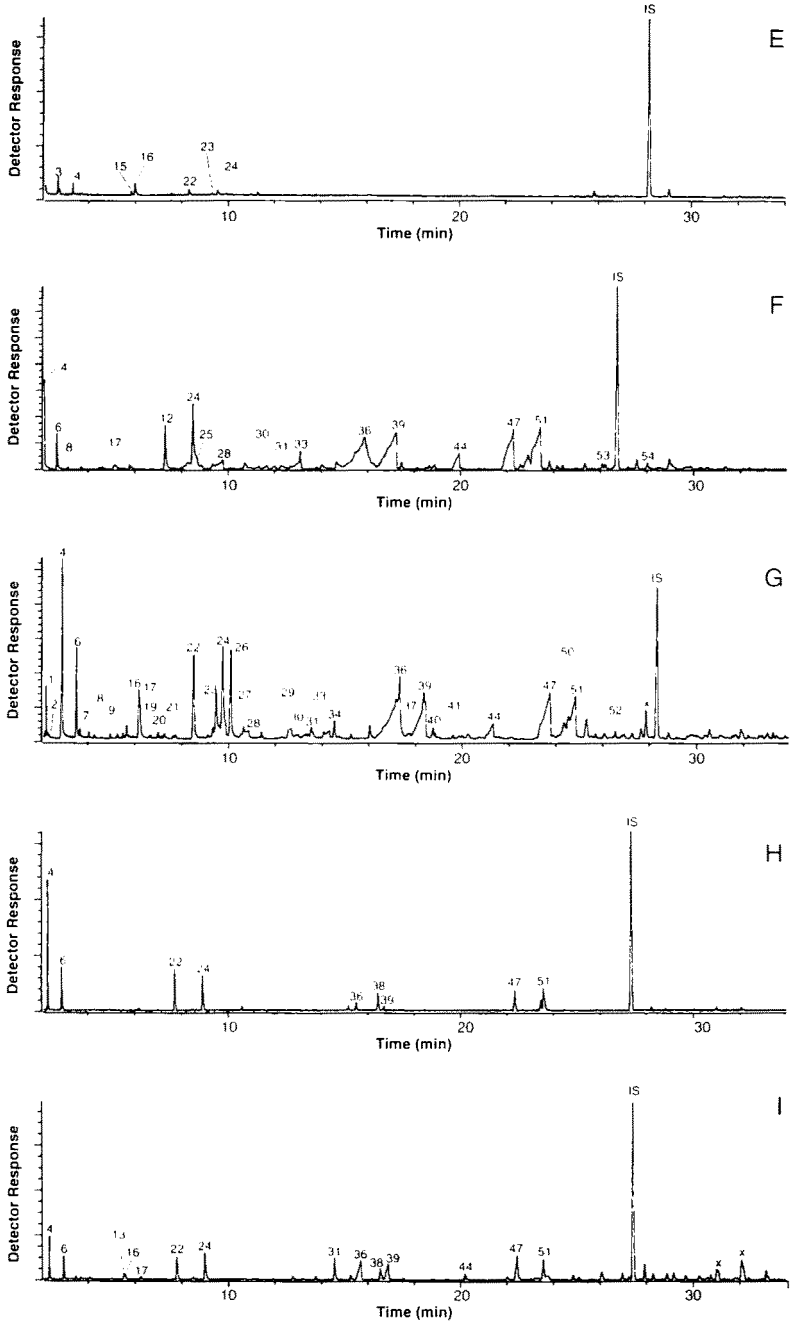

FIG. 2. Continued.

TABLE 1. COMPOUNDS IDENTIFIED IN ANAL GLAND SECRETION OF MOLE
(Numbers in parentheses match those in Fig. 2.)

Carboxylic acids
- Butanoic acid (10)[a]
- 2-Methylbutanoic acid (18)
- Pentanoic acid (28)[a]
- Heptanoic acid (33)[a]
- Octanoic acid (39)[a]
- Nonanoic acid (44)[a]
- 6-Methylnonanoic acid (47)
- Undecanoic acid (53)[a]
- 3-Methylbutanoic acid (14)[a]
- 4-Methylpentanoic acid (25)[a]
- Hexanoic acid (30)[a]
- 7-Octenoic acid (36)
- 2-Methylhexanoic acid (40)
- 8-Nonenoic acid (45)[a]
- Decanoic acid (51)[a]
- Dodecanoic acid (54)[a]

Esters
- Methyl 2-ethylpropenoate (15)
- Ethyl decanoate (48)[a]
- Methyl decanoate (42)

Aldehydes
- Pentanal (5)
- Heptanal (20)[a]
- Nonanal (34)[a]
- Hexanal (8)[a]
- Octanal (47)[a]

Ketones
- 3-Hydroxybutan-2-one (1)
- Heptan-2-one (19)[a]
- Nonan-2-one (32)

Ketones Continued
- Tridecan-7-one (46)
- 5-Methylhexan-2-one (13)[a]
- 11-Dodecen-2-one (29)
- Undecan-2-one (41)
- Pentadecan-8-one (52)

Alcohols
- Pentan-2-ol (2)
- Pentan-1-ol (6)[a]
- Hexan-2-ol (9)[a]
- 3-Methylpentan-1-ol (12)[a]
- Hexan-1-ol (17)[a]
- 3-Methylhexan-1-ol (22)
- Heptan-1-ol (24)[a]
- Octan-1-ol (31)[a]
- *cis*(?)-3-Nonen-1-ol (37)
- Decan-1-ol (43)[a]
- 2-Undecen-1-ol (50)
- 3-Methylbutan-1-ol (4)[a]
- 3-Methylbut-2-en-1-ol (7)
- 4-Methylpentan-1-ol (11)[a]
- 5-Methylhexan-2-ol (16)
- Heptan-2-ol (21)
- *cis*-3-Hepten-1-ol (23)[a]
- *cis*(?)-3-Octen-1-ol (26)
- 1-Octen-3-ol (35)[a]
- Nonan-1-ol (38)[a]
- 3-Decen-1-ol (49)

Sulfide
- Dimethyl disulfide (3)

[a]Compounds whose identities were confirmed by comparison of retention times with those of the authentic compounds.

adult moles. Bacterial activity has been shown to be responsible for volatile carboxylic acids in the anal sacs of the lion (Albone et al., 1974), the red fox (Albone et al., 1974; Ware and Gosden, 1980), and the beaver (Svendsen and Jollick, 1978) and in the inguinal pouch of the rabbit (Merritt et al., 1982), but we have shown (Buglass et al., 1995) that no bacteria are present in the anal gland of the mole, and so carboxylic acids are formed by metabolic processes within the cells of the animal.

Bearing in mind that the samples came from different animals, the chromatograms in Figure 2A and B show remarkable similarity in the profile and composition of the volatile components of the anal gland secretion of the male

in the nonbreeding and the breeding seasons. The secretions are characterized by relatively high concentrations of carboxylic acids. The secretion from the nonbreeding female (Figure 2D) is also clearly similar in composition to that of the male, showing large carboxylic acid peaks. This corroborates the other male-like characteristics of the nonbreeding female, such as her external genitalia. As the female approaches estrus, however, there are profound changes in the composition of the anal gland secretion (Figure 2E), which occur together with the recession of the glands (Figure 1). The carboxylic acids disappear relatively early in the process and, as a consequence, alcohols become the main components for the period during which the glands are still secreting. The pattern in the female secretion is repeated in the rare occurrence of second estrus in September (Figure 2H), but in this case the carboxylic acids had not entirely disappeared at the time the animal was trapped. The characteristic male-like composition of the female secretion is quickly reestablished after estrus, as is shown in the secretion of the pregnant and lactating female (Figure 2F and G, respectively), both of which have significant carboxylic acid peaks.

The seasonal changes in the size of the female anal gland and in the composition of its secretion occur concurrently with profound changes in the ovary. The ovary contains two distinct glandular masses: an ovarian part and an interstitial gland, and these features vary in relative size and activity during the year. Outside the breeding season the interstitial gland is far larger than the ovarian part; but during the breeding season the ovarian part enlarges as the interstitial part atrophies, and for a short period the ovarian part is the larger of the two glandular masses (Harrison Matthews, 1935). The enlargement of the ovarian part occurs during the period in which the anal glands regress. After parturition the interstitial gland begins to return to its dominant state as the ovarian part atrophies. The interstitial gland is considered to be homologous to the male testis and to produce male hormones that suppress the normal female characteristics outside the breeding season (Harrison Matthews, 1935). The return to dominance of the interstitial tissue after parturition coincides with the reappearance of the male-like composition of the female anal gland secretion.

Our results provide further evidence for the role of the anal gland secretion in territorial maintenance. Outside the breeding season, when both sexes deter conspecifics from entering their tunnel systems, the glandular secretions of both sexes are clearly similar in their volatile compositions, which suggests that they produce a common chemical "keep out" signal. For female moles this signal is interrupted only in the breeding season with regression of the anal glands, often to the point of invisibility (Figure 1). It is at this time that a male is able to approach the female and mate. The change in composition of the secretion that accompanies the regression of the gland may produce a chemical signal that alerts a neighboring male mole to the approach of sexual receptiveness in the female, and complete cessation of the secretary activity as regression of the

glands is completed may serve to fine tune the mating period; however, a note of caution is required here, for the amount of secretion produced by the regressing glands is very small. It may simply be that males are no longer deterred from entering the female tunnels owing to the switching off of a keep-out signal; moreover, our results do not preclude the attraction of males by odors from other sources in the receptive female. The rapid reappearance of the male-like composition of the female secretion after estrus is consistent with the need for the female to protect her tunnel systems during pregnancy, and particularly during lactation, in order to ensure the young's survival.

The analytical data suggest that carboxylic acids play a significant semiochemical role. A set of identical carboxylic acids form the dominant component in the secretion of adult males and anestrous females; it is these carboxylic acids that disappear in the secretion of the proestrous female; they are rapidly reestablished during pregnancy and lactation. The development in the composition of the secretion of juveniles is also instructive. Our analysis showed that the composition of the secretion of both sexes at the same stage of maturity was very similar and that the composition of the secretion changes as the animal develops and its anal glands increase in size and secretory ability. Figure 2C and 2I illustrate this trend. In the young male caught in May (Figure 2C), carboxylic acids are only very minor components, and the profile is similar to those of the proestrous females (Figures 2E and 2H). Figure 2I shows the chromatogram from a juvenile female caught in late July. This animal was more mature than the juvenile male, although her reproductive tract was still not fully developed. The animal would be nearing the dispersal phase when she would need to set up a territory and mark it as her own. The carboxylic acids in the secretion have now become significant components at this stage in the mole's development.

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PHYTOTOXICITY OF CACALOL AND SOME DERIVATIVES OBTAINED FROM THE ROOTS OF *Psacalium decompositum* (A. GRAY) H. ROB. & BRETTELL (ASTERACEAE), MATARIQUE OR MATURIN

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Abstract—The effect of cacalol and extracts (water and petroleum ether) derived from the roots of *Psacalium decompositum* (Asteraceae) on the germination and radicle growth of two plants, *Amaranthus hypochondriacus* (Amaranthaceae) and *Echinochloa crusgalli* (Poaceae), and on the radial growth of four phytopathogenic fungi is described. The activity of two cacalol derivatives (methyl cacalol and cacalol acetate) was also investigated. Germination of *A. hypochondriacus* was inhibited by almost all the treatments. The extracts and cacalol produced a significant inhibition of radicle growth of *A. hypochondriacus* and *E. crusgalli*. Cacalol acetate showed a specific inhibition on *E. crusgalli*, and methyl cacalol significantly inhibited the growth of *A. hypochondriacus*. In general, antifungal activity depended upon the target fungi and the concentration of each treatment. Cacalol had also effects on the morphology and coloration of the fungal mycelium. The bioactivity shown by the extracts of *Psacalium decompositum* on the tested seeds and fungi is mainly due to their content in cacalol.

Key Words—*Psacalium decompositum*, Asteraceae, sesquiterpene, cacalol, phytotoxicity, fungitoxicity, phytopathogenic fungi, *Amaranthus hypochondriacus*, Amaranthaceae, *Echinochloa crusgalli*, Poaceae.

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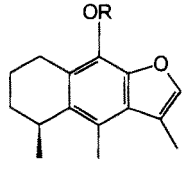
INTRODUCTION

Psacalium decompositum (A. Gray) H. Rob. & Brettell (Asteraceae), locally called matarique or maturin, is a shrub widely distributed in the northern part of Mexico. With three other species of the same genus—*P. peltatum*, *P. sinuatum*, and *Psacalium* sp., and the species *Acourtia thurberi*—all of which belong to the Asteraceae family and are native of Mexico and southern North America, *P. decompositum* is part of a medicinal plant complex widely used in this area. All species of the complex share similar names and root characteristics, and all are used as remedies for the same illnesses. The infusion of the roots of *P. decompositum*, the most valued plant of the group, has a pungent odor and a bitter hot taste; it is used for the treatment of rheumatism, colds, stomach ache, back pains, jaundice, malaria, diabetes, and snake bites. It is also employed as a tonic and diuretic agent (Linares and Bye, 1987).

Romo and Joseph-Nathan (1964) isolated from the roots of *P. decompositum* two crystalline sesquiterpenes named cacalol (Figure 1a) and cacalone. These compounds were the first representatives of a new class of molecules possessing the unusual tetrahydronaphthofuran structure, not previously known to occur in nature (Figure 1a). Cacalol derivatives were also isolated from *Senecio* species and from *Roldana heterogama* (Pippen, 1968; Bohlmann and Zdero, 1978; Bohlmann and Bapuji, 1982; Bohlmann et al., 1990).

Terpenoids, the largest group of plant chemicals (15,000–20,000) with a common biosynthetic origin, possess enormous potential for mediating ecological interactions (Langenheim, 1994). Plant terpenoids may produce effects on seeds and soil microbiota through volatilization, leaching from plants, or decomposition of plant debris. These interactions can significantly affect community and ecosystem properties (Harper, 1977; Duke, 1991). The allelopathic and phytotoxic properties of plant terpenoids, particularly sesquiterpene lactones, have received special attention in numerous publications and reviews (Amo and



1a, R = H
1b, R = COMe
1c, R = Me

FIG. 1. Chemical structures of cacalol (1a), cacalol acetate (1b), and methyl cacalol (1c).

Anaya, 1978; Picman, 1986; Fischer, 1991; Macías et al., 1992; Fischer et al., 1994). Earlier treatments of phytotoxic and possible allelopathic actions of secondary plant metabolites including terpenoids were presented in several books by Rice (1984), Thompson (1985), Putnam and Tang (1986), Waller (1987), and Harborne (1988).

Lotina-Hennsen et al. (1991) found that cacalol inhibited ATP synthesis, proton uptake, and electron transport (basal, phosphorylating, and uncoupled) from water to methylviologen. Cacalol and its derivatives act as electron transport inhibitors at the oxygen evolution level. Taking these results as a basis, Lotina-Hennsen et al. (1991) suggested that cacalol probably behaves as an allelochemic agent, interfering with the metabolism of other organisms. In addition, Jiménez-Estrada et al. (1992) found that the organic extracts of the roots, cacalol, and some of its derivatives had antimicrobial activity against *Candida albicans*, *Cryptococcus neoformans*, *Staphylococcus aureus*, and *Streptococcus pyogenes*.

These previous studies clearly show that secondary metabolites from the roots of this plant possess biological activity. Therefore, as part of a project devoted to the search of bioactive metabolites from Mexican plants of medicinal or ecological importance and with potential agrochemical applications, we studied the effect of some extracts of the roots of *P. decompositum*, as well as cacalol and some of its derivatives on the germination and growth of two species of plants and four species of phytopathogenic fungi.

METHODS AND MATERIALS

General Bioassay Procedures

Bioassays with Seeds. The roots of *Psacalium decompositum* were obtained in October 1992, at the Sonora Market, the biggest medicinal plant market of Mexico City. The whole dry roots were soaked for 4 hr in distilled water (1 g/100 ml). The water extract was filtered, and the osmotic pressure was measured in a freezing-point osmometer in order to avoid the effect of this factor on germination and growth (Anaya and Rovalo, 1976). Osmotic pressures of 15–20 mosm/liter were recorded, well below the level of 150 mosm/liter observed to delay germination (Leather and Einhellig, 1985). Bioassay experiments were designed to measure the effect of the water extract (1%) and petroleum ether extract of the roots of *P. decompositum*, and of cacalol, methyl cacalol, and cacalol acetate (50, 100, and 200 μg/ml—because of the limiting available amounts of the compounds) on the radicle growth of *Amaranthus hypochondriacus* (Amaranthaceae) and *Echinochloa crusgalli* (Poaceae).

A completely randomized block design with four repetitions × treatment

for each target species was used. Ten seeds of each species were sown on Whatman No. 4 qualitative filter paper in 5-cm Petri dishes. Distilled water (1.5 ml) was added in each dish. The dishes were incubated in darkness at 27°C. The radicle length was measured after 24 hr in the case of *A. hypochondriacus*, and 48 hr in the case of *E. crusgalli*. The data were analyzed by ANOVA.

Bioassay with Phytopathogenic Fungi. Four target species were chosen: *Helminthosporium* sp., *Fusarium oxysporum*, *Alternaria* sp., and *Pythium* sp. The bioassays were carried out by incubating in 10-cm Petri dishes a fungal bud on a potato–dextrose–agar (PDA) sterile medium, to which the water extract of *P. decompositum* (1%) or the petroleum ether extract or the pure compounds (5, 50, and 500 μg/ml) were added. Buds 5 mm in diameter were obtained from pure 8-day-old fungal cultures. The sown fungi were incubated in darkness at 27°C. A randomized complete block design with three replications was utilized. The effect of compounds was determined by measuring the radial growth of the mycelium. Two perpendicular measurements of colony diameter were taken, and the mean value was obtained. The measurements were made at three and eight days. The data were analyzed by ANOVA.

CHEMICAL PROCEDURES

Isolation of Cacalol

Five hundred grams of dry roots of *P. decompositum* were extracted three times with 1 liter of petroleum ether (PE). The extract was evaporated under reduced pressure to dryness, yielding 45 g of a red residue with a characteristic odor. Ten grams of this residue was chromatographed through an alumina column (400 g). Elution was accomplished with PE and with PE–AcOEt (9.8–0.2). From several of these column fractions, crude cacalol was obtained as a yellow crystalline product. Cacalol was purified by recrystallization from PE and PE-pentane to yield 350 mg of pure compound **1a** (Figure 1), as prisms mp 92–94°, negative $FeCl_3$ test $[\alpha] + 10°$, λ_{max} 218, 256, 264, and 286 nm; ϵ 30,400, 10,500, 10,000, 1840; ν max 3550 (hydroxyl group), weak bands at 1635 and 1602 cm^{-1}. ^1H NMR δ ppm: 1.2 (d, 3H, C-12), 2.3 (d, 3H, C-10), 2.5 (s, 3H, C-11), 5.0 broad (1H, OH), 7.2 (1H, C-2), 2.9 (q, 1H, C-5), 1.8 (m, 4H, C-6 and C-7), and 3.2 (m, 2H, C-8). Found: C, 78.23; O, 14.17. Calc for $C_{15}H_{18}O_2$: C, 78.23; H, 7.88; O, 13.89%. MS, M^+ 230. Fragments *m/z* 215, 200, and 187 (Jiménez-Estrada et al., 1992).

Cacalol Acetate. Acetylation of 40 g of the brown–red oil from the PE extract was made by adding acetic anhydride and pyridine in the usual way (1 hr on a steam bath). This procedure produced 33 g of a red oil, which was chromatographed in a column with bentonite (tonsil) as the adsorbent and PE

as the eluent; pale yellow crystals (19%) of cacalol acetate (Figure 1b) were obtained. mp = 98° and R_f = 0.72 (8:2 petroleum ether–ethyl acetate).

Methyl Cacalol. Cacalol (800 mg) was dissolved in methanol (20 ml) and treated with an ethereal solution of diazomethane (prepared from 3 g of *N*-nitrosomethylurea). After 24 hr the excess diazomethane was eliminated with acetic acid and the solution evaporated to dryness. After an alumina column chromatography (PE), the oily residue (730 mg) produced yellow crystals identified with IR, NMR and MS as methyl cacalol (Figure 1c) (Jiménez-Estrada et al., 1992).

RESULTS AND DISCUSSION

Bioassays with Seeds. The two target species, *A. hypochondriacus* and *E. crusgalli* were chosen because of their experimental convenience (their seeds are small and have a high and quick germination) and represent the two major groups of angiosperm, monocotyledons and dicotyledons. Most phytotoxic compounds and herbicides show a selective activity on these two groups of plants. On the other hand, *A. hypochondriacus* belongs to a family with some remarkable weeds such as *A. dubius*, *A. retroflexus*, *A. spinosus*, and *A. hybridus*. Two cultivated amaranths, *A. hypochondriacus* (sin *A. leucocarpus*) and *A. cruentus*, are considered as pseudocereals with great nutritional content (particularly proteins) and had a great economic importance in pre-Hispanic agriculture in the Valley of Mexico (Ritchie, 1979; Amo et al., 1988). These two cultivated species can easily escape into the wild and become important weeds in different field crops (Anaya et al., 1987). *E. crusgalli* is a weed grass with a wide geographic distribution and is considered as one of the main weeds in a number of crops (Duke, 1985).

The effects of the different treatments from *P. decompositum* on the germination and radicle growth of the two target species are shown in Table 1. Although there is natural variation in the germination of the test seeds, particularly *E. crusgalli*, it is clear that germination of *A. hypochondriacus* was affected by all the treatments, notably by the water extract and cacalol. On the other hand, germination of *E. crusgalli* was affected only by cacalol at 200 μg/ml.

The water and petroleum ether extracts and cacalol produced a significant inhibition of the radicle growth of both species tested. The IC_{50} values for the petroleum ether extract and cacalol on *A. hypochondriacus* were 143.58 and 102.73 μg/ml, respectively. In the case of *E. crusgalli* the IC_{50} values were 218.43 and 266.43 μg/ml, respectively.

Lotina-Henssen et al. (1991) suggested that the —OH group of cacalol is an important structural feature for the inhibition of oxygen evolution in photosynthesis. Therefore, in order to investigate whether the free hydroxyl group of

TABLE 1. EFFECTS OF WATER EXTRACT, PETROLEUM ETHER EXTRACT, AND PURE COMPOUNDS FROM ROOTS OF *P. decompositum* ON GERMINATION AND RADICLE GROWTH OF *A. hypochondriacus* AND *E. crusgalli*

Treatments	*Amaranthus hypochondriacus*		*Echinochloa crusgalli*	
	Germination (%)	Inhibition of radicle growth (%)	Germination (%)	Inhibition of radicle growth (%
Control	92.5	0	75	0
Water extract, 1%	50"	37"	75	54"
Petroleum ether extract (μg/ml)				
50	77.5"	18.5	85	28.2"
100	60"	47.8"	67.5	43.8"
200	67.5"	55.4"	62.5	46.5"
Cacalol (μg/ml)				
50	57.5"	44"	65	31.4"
100	67.5"	41.2"	65	49.0"
200	37.5"	63.6"	60"	43.5"
Cacalol acetate (μg/ml)				
50	80	1.9	65	28.6"
100	67.5"	3.8	75	25.3"
200	67.5"	5.7	77.5	36.3"
Methyl cacalol (μg/ml)				
50	62.5"	21.8"	72.5	2.1
100	72.5"	18.9	85	2
200	70"	22.3"	67.5	30.1"

"$P < 0.05$.

cacalol has any influence on the activities we have observed, we synthesized two derivatives of greater liposolubility than cacalol: cacalol acetate (Figure 1b), and methyl cacalol (Figure 1c). Cacalol acetate significantly inhibited the radicle growth of *E. crusgalli* but did not affect growth of *A. hypochondriacus*. On the other hand, the inhibitory effect of methyl cacalol on *E. crusgalli* was observed only at 200 μg/ml. When acting on *E. crusgalli*, methyl cacalol was less potent than cacalol and cacalol acetate in inhibiting radicle growth. To a lesser degree and independently of concentration, methyl cacalol inhibited the growth of *A. hypochondriacus*.

These results clearly indicated that substitution of the free hydroxyl group in cacalol did not enhance the inhibition of radicle growth in *A. hypochondriacus* and *E. crusgalli*, but resulted in a more selective activity. It is possible that cacalol acetate, because of its ester properties, could be biotransformed to cacalol by enzymatic action inside the seeds of *E. crusgalli*. On the other hand, *A.*

hypochondriacus may be unable to make this biotransformation, so cacalol acetate remains as such inside its seeds, causing no effect on them. Methyl cacalol acts in an analogous, but less potent fashion than cacalol acetate.

Bioassays with Phytopathogenic Fungi. The four phytopathogenic species chosen for these bioassays have economic importance. They produce different common diseases in plants, especially cultivated ones, affecting leaves, stems, flowers, fruits, and tubers (Agrios, 1991).

The effects of the water extract from the roots of *P. decompositum* on the radial growth of the phytopathogenic fungi are shown in Figure 2. There was a specific effect of the water extract on each fungus species. A significant stimulant effect of the water extract on the growth of *Alternaria* at three days of growth was found. Conversely, this treatment exerted a significant inhibitory effect on the growth of *Pythium* and *Fusarium*. At eight days a significant inhibition was observed for *Pythium* and *Helminthosporium*, and at that time, no stimulant effect on the other fungi was observed. This inhibition could prove to be useful in plant protection (El-Zik and Frisbie, 1985).

The same phenomenon was observed in the bioassays with the petroleum ether extract, cacalol, and its derivatives. Their effects on radial growth of fungi at three and eight days of growth are shown in Table 2. At 500 μg/ml, the petroleum extract showed greater inhibition than the other treatments. At this concentration, this extract strongly inhibited the growth of *Pythium* after three

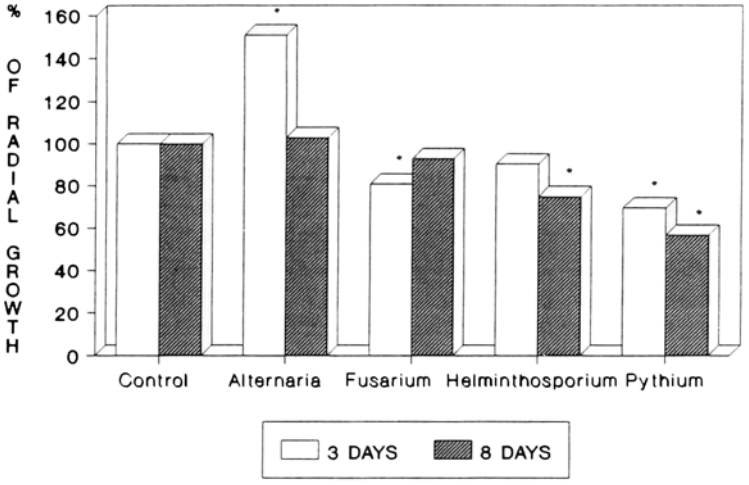


FIG. 2. Effects of water extract of the roots of *Psacalium decompositum* on the radial growth of phytopathogenic fungi at three and eight days of growth.

TABLE 2. EFFECT OF PETROLEUM ETHER EXTRACT, CACALOL AND DERIVATIVES FROM THE ROO OF *Psacalium decompositum* ON THE RADIAL GROWTH (%) OF PHYTOPATHOGENIC FUNGI AT 3 AND 8 DAYS OF GROWTH

	Pythium		*Alternaria*		*Fusarium*		*Helminthosporiu*	
Treatments	3 days	8 days	3 days	8 days	3 days	8 days	3 days	8 day
Control	100	100	100	100	100	100	100	100
Petroleum ether extract (μg/ml)								
5	139[a]	107	111.6[a]	132.3[a]	107.3	132.8[a]	134.8[a]	148.3
50	77.8[a]	64.9[a]	87[a]	93.7	96.1	115.4[a]	92.9	94.2
500	32.4[a]	20.3[a]	48.9[a]	48.1[a]	45.1[a]	50.8[a]	52.9[a]	55.2
Cacalol (μg/ml)								
5	80[a]	170[a]	110.3	110.2	91	94.4	109.3	125.7
50	54.7[a]	113.6[a]	88.3[a]	85.1[a]	83[a]	80.9[a]	104.4	107.4
500	40[a]	66.6[a]	81[a]	58.2[a]	68.8[a]	68.3[a]	86.6[a]	88.6
Methyl cacalol (μg/ml)								
5	121.1[a]	143.6[a]	91.1	86.9[a]	90.4	106.2	98.7	99.4
50	114.2[a]	130.3[a]	85.2[a]	77.7[a]	97.4	106.6	88.2[a]	88.7
500	96	100.9	72.6[a]	68.1[a]	98.3	105.9	90.7	90.1
Cacalol acetate (μg/ml)								
5	106	109.3	94.1	96.8	93.9	94.2	116.8[a]	125.5
50	99.5	98	93.2	95.9	92.6	97.9	109	111.8
500	99.2	100.7	78.2[a]	78.3[a]	88.3[a]	98.5	86.6[a]	89[a]

[a] $P < 0.05$.

and eight days of growth. At a lower concentration (5 μg/ml), this extract produced a significant stimulant effect on the growth of *Pythium* after three days of growth. A similar effect of the petroleum ether extract was observed on the growth of the other species of fungi. The fungitoxic effect of the petroleum ether extract at 500 μg/ml was stronger than the effect observed with the other treatments at the same concentration, probably due to a synergistic effect of the chemicals in the mixture where cacalol is the major compound.

After three days of growth cacalol significantly inhibited the radial growth of *Pythium* at the three concentrations tested (Table 2). At this time, the inhibitory effect of cacalol increased when the concentration was changed from 50 to 500 μg/ml. At eight days of growth, the inhibitory effect of cacalol at 500 μg/ml on the growth of *Pythium* is still significant, but its effect at this time is totally the opposite at 5 and 50 μg/ml. Cacalol produced a significant inhibition on the growth of *Alternaria* and *Fusarium* after three and eight days of growth at 50 and 500 μg/ml. To a lesser degree, cacalol has an inhibitory effect on *Helminthosporium* only at 500 μg/ml.

Methyl cacalol (Figure 1c) showed no inhibitory effect on the growth of *Pythium* at any time (Table 2) but produced a significant stimulation on the growth of *Pythium* at 5 and 50 μg/ml after three and eight days of growth. *Alternaria* was the most sensitive species to the effect of methyl cacalol. It was significantly inhibited by this compound at the three concentrations tested, particularly after eight days of growth. Methyl cacalol had no effect on the growth of *Fusarium* and inhibited *Helminthosporium* only at 50 μg/ml.

Cacalol acetate (Figure 1b) was the least active compound. It had no effect on the growth of *Pythium*; however, it slightly inhibited *Alternaria* and *Fusarium* at 500 μg/ml after three and eight days of growth. The effect of cacalol acetate on *Helminthosporium* depended on concentration; it was stimulatory at 5 and 50 μg/ml and slightly inhibitory at 500 μg/ml.

In general, the fungitoxic activity of all treatments used in the bioassays was concentration-dependent, a fact usually observed with some allelochemicals and other growth regulators. Many secondary metabolites have a stimulatory effect at low concentrations and an inhibitory one at higher concentration (Einhellig, 1985). Results of our bioassays with fungi confirm this fact. *Pythium* was the most sensitive species to the fungitoxic effect of these treatments. On the other hand, it is probable that all these fungi could metabolize the four treatments tested at the lower concentrations and use them as a carbon source (Griffin, 1994). Our interest was focused on the effect of the different treatments on the growth of the mycelium, but probably more relevant than this was the effect of cacalol on the morphology and structure of the mycelium of the target fungi, which changed both their appearance and color in the presence of the compound. It is possible that this sesquiterpene modifies some aspects of the metabolism and reproduction of these particular microorganisms. Overall we can conclude that the bioactivity exhibited by the extracts of the roots of *Psacalium decompositum* on the tested seeds and fungi is mainly due to their cacalol content.

It is important to point out the potential ecological role of the allelochemicals produced by the roots of *Psacalium decompositum* and other related plants. We have not done any field studies yet. However, further research under natural conditions is in progress in order to determine whether water can leach the mixture of allelochemicals from the roots of this species, and if the released mixture has an effect on other plants and microorganisms in the environment. If cacalol and other related secondary metabolites are effectively released in sufficient amounts to the environment, they could have an ecological effect on other receptor organisms, particularly on rhizosphere microorganisms and surrounding plants. Other related species such as *Senecio* spp., *Roldana heterogama*, *Psacalium peltatum*, and *P. sinuatum* are known to synthesize cacalol derivatives. Therefore, it would be important to study the allelochemical role of these secondary metabolites.

Increasing global concern about the indiscriminate use of, overdependence on, and hazards of synthetic pesticides has prompted exploration of natural plant products as alternatives for plant protection since they could be expected to be comparatively safe. Surprisingly, while much emphasis has been given to the integration of various strategies of pest control, little effort appears to have been made to develop multipurpose natural pesticides capable of controlling more than one agricultural pest (Rizvi and Rizvi, 1992). The present study points out the potential of allelochemicals from the roots of *P. decompositum* as natural selective herbicides and fungicides. We are well aware that traditional ethnobotanical knowledge, so rich in Mexico, has frequently led to the discovery of bioactive compounds. Thus, we will continue to explore Mexican biodiversity to expose the great chemical diversity and ethnobiological richness (Anaya et al., 1995).

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PURIFICATION AND CHARACTERIZATION OF NEW FATTY ACIDS WITH ANTIBIOTIC ACTIVITY PRODUCED BY *Sporothrix flocculosa*[1]

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Abstract—Chloroform extracts from a culture broth of the biocontrol fungus *Sporothrix flocculosa* were separated by thin-layer chromatography (TLC). Compounds with antifungal activity were revealed with the *Cladosporium cucumerinum* bioassay on TLC plates and were isolated by using silica gel column, preparative TLC, and reverse-phase high-performance liquid chromatography (RP-HPLC). They were identified by standard methods of nuclear magnetic resonance (NMR), gas chromatography (GC), mass spectrometry (MS), and infrared spectrum (IR). In addition to an active molecule previously described, two new fatty acids with antibiotic activity were characterized, 9-heptadecenoic acid and 6-methyl-9-heptadecenoic acid, the latter displaying the strongest activity among all three active molecules. This high number of active metabolites produced by *S. flocculosa* would explain its rapid and strong activity as a biocontrol agent of powdery mildews. In this context, screening of *S. flocculosa* isolates for increased production of these molecules should help in selection of candidates with superior biocontrol properties.

Key Words—*Sporothrix flocculosa*, antibiotics, fatty acids, biocontrol.

INTRODUCTION

Sporothrix flocculosa (Traquair, Shaw & Jarvis) has been reported in numerous studies as a potential antagonist against powdery mildew fungi (Jarvis et al., 1989; Hajlaoui and Bélanger, 1991, 1993; Bélanger et al., 1994). Practical exploitation of *S. flocculosa* as a biocontrol agent presupposes a thorough knowl-

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edge of its mode of action. Only through an understanding of the mechanisms of activity of *S. flocculosa*, will it be possible to achieve consistency of efficacy and selection of superior strains. To this end, cytochemical studies of the interaction between *S. flocculosa* and *Sphaerotheca pannosa* var. *rosae* have revealed that disintegration of host cytoplasm occurred in the absence of hyphal penetration, suggesting the involvement of extracellular diffusible molecules (Hajlaoui et al., 1992). In addition, it was shown that *S. flocculosa* produced antimicrobial compounds active against several microorganisms such as *Fusarium oxysporum*, *Botrytis cinerea*, *Trichoderma viride*, and *Bacillus subtilis* (Hajlaoui et al., 1994; Choudhury et al., 1994). Recently, two aliphatic molecules (4-methyl-7,11-heptadecadienoic acid and 4-methyl-7,11-heptadecadienal), were isolated from liquid cultures of *S. flocculosa* and *S. rugulosa*, with the former compound displaying antimicrobial activity (Choudhury et al., 1994). It thus appears evident that antibiosis is the main mode of action by which *S. flocculosa* exerts its biocontrol activity. However, it is still unclear whether this activity is mediated by one or several molecules, and if the latter, whether those molecules can be isolated and characterized. In this context the objectives of the present study were twofold: (1) to screen extracellular metabolites produced by *S. flocculosa* for potential antifungal activity, and (2) to purify and characterize active molecules.

METHODS AND MATERIALS

Fungal Culture. Single-spore isolates of *Sporothrix flocculosa* were obtained by reisolating the fungus from rose leaves that were submitted to weekly applications of a fungal suspension initiated from an isolate of *S. flocculosa* graciously provided by Dr. W. R. Jarvis, Agriculture and Agri-food Canada (Harrow, Ontario, Canada). Stock cultures of the isolates were maintained at 4°C on yeast–malt–peptone–dextrose agar (YMPDA) containing yeast extract (3 g/liter), malt extract (3 g/liter), peptone (5 g/liter), dextrose (10 g/liter), and bacto-agar (15 g/liter). Erlenmeyer flasks (500 ml) each containing 100 ml YMPD broth were inoculated with 10^6 conidia/ml of *S. flocculosa*. The fungus was grown in still culture at 26°C in the dark for 28 days.

Cladosporium cucumerinum Ellis and Arth was obtained from Biosystematics Research Centre (Ottawa, Ontario, Canada) and was maintained on potato dextrose agar (PDA) at 25°C.

Screening of Molecules with Antifungal Activity. The liquid medium was separated from the biomass through a filter paper (Whatman No. 1) and the mycelium discarded. The filtrates were further extracted with chloroform ($CHCl_3$). The organic fraction was dehydrated with Na_2SO_4 and dried under vacuum. The residue was redissolved in chloroform.

Thin-layer chromatography (TLC) of the culture filtrate extracts was carried out on silica gel plates (Merck 60 F 254, 0.1 mm thick, 10 × 5 cm). The extracts were deposited as spots of 80 μl, and after drying, the chromatograms were developed in ethyl acetate–hexane (1 : 4, v/v). The spots were viewed under ultraviolet radiation at 254 nm and 366 nm. To localize zones of antifungal activity, silica gel plates were seeded with a sporal suspension of *Cladosporium cucumerinum* as described by Homans and Fuchs (1970). Because of the dark pigmentation of spores and mycelium of this fungus, zones of antifungal activity can be readily identified as white spots, each one representing zones where spore germination is inhibited.

Purification Procedures. The chloroform extracts from the filtrates were applied to a silica gel column (Silica Gel; 230–400 mesh, Baker Analyzed reagent) and flash-chromatographed by successive elution with 100% hexane (200 ml); 150 ml each of 1%, 5%, 8%, and 15% of diethyl ether in hexane; 250 ml of 100% diethyl ether; and 100% of methanol. For each eluted fraction, assessment of antifungal activity was carried out with the TLC bioassay as described above. The active fractions were purified with a combination of preparative TLC and high-performance liquid chromatography (HPLC). Preparative TLC was performed with Silica Gel 60 G (0.30 mm thick). Chloroform concentrates from the active fractions were spotted in a line along the bottom of the plate and developed in ethyl acetate–hexane (1 : 4, v/v). The bands corresponding to active spots were scraped off the plates, and the active molecules were extracted from silica gel with chloroform. These chloroform extracts were separated by HPLC with C_{18} reverse-phase columns (Millipore-Waters, 8 × 10 cm). Elution was performed with methanol–water–acetonitrile in gradient mode at a flow rate of 1.0 ml/min. Absorbance of the effluent was monitored by a photodiode array scanning between 220 and 400 nm.

Identification of Active Fractions. FT-IR spectra of the fractions were obtained on a Bruker FT-IR instrument. All NMR spectroscopy (^1H, ^{13}C, 2D, Hector) were recorded in deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) as an internal standard using a Bruker 300 MHz. GC-MS data were recorded with a Hewlett Packard (HP) 5890 series II gas chromatograph coupled with MS HP mass selective detector 5972. Precise location of double bonds was obtained by ozonolysis using O_3/HCO_2H according to the method described by Frans et al. (1965). The position of methyl branching was confirmed by using acylpyrrolidines according to Andersson and Holtman (1975).

RESULTS AND DISCUSSION

On silica gel thin-layer chromatograms, chloroform extracts were separated into several spots as visualized under ultraviolet radiation at 254 and 366 nm.

Spraying of a sporal suspension of *C. cucumerinum* revealed only one major active spot in the chloroform extract with an approximate R_f value of 0.8 (Figure 1). However, when the active spot was viewed under low (254 nm) and high ultraviolet (366 nm) radiation, further bands were observed, indicating the presence of several molecules at this spot.

Fractionation of the chloroform extract by column chromatography and preparative TLC followed by reverse-phase HPLC revealed three active compounds on TLC based on the bioassay with *C. cucumerinum* (Figure 2). The physicochemical analysis (^{1}H NMR, ^{13}C NMR, IR, GC-MS) of these compounds revealed two new active fatty acids: 6-methyl-9-heptadecenoic acid ($C_{18}H_{34}O_2$) (Figure 3a) and 9-heptadecenoic acid ($C_{17}H_{32}O_2$) (Figure 3b) and another active compound corresponding to 4-methyl-7,11-heptadecadienoic acid previously isolated and characterized by Choudhury et al. (1994). Based on our bioassays, the 6-methyl-9-heptadecenoic acid appeared to display the highest antifungal activity among the three isolated compounds (Figure 2).

FIG. 1. Fungitoxicity of chloroform extracts from *Sporothrix flocculosa* culture filtrates on thin layer chromatograms (TLC). Eighty microliters were spotted on a silica gel TLC plate and developed with ethyl acetate–hexane (1:4, v/v). After drying, the plate was sprayed with a conidial suspension of *Cladosporium cucumerinum* and incubated at room temperature. After 48 hr of incubation, one major zone of inhibition was revealed with R_f value of approximately 0.8.

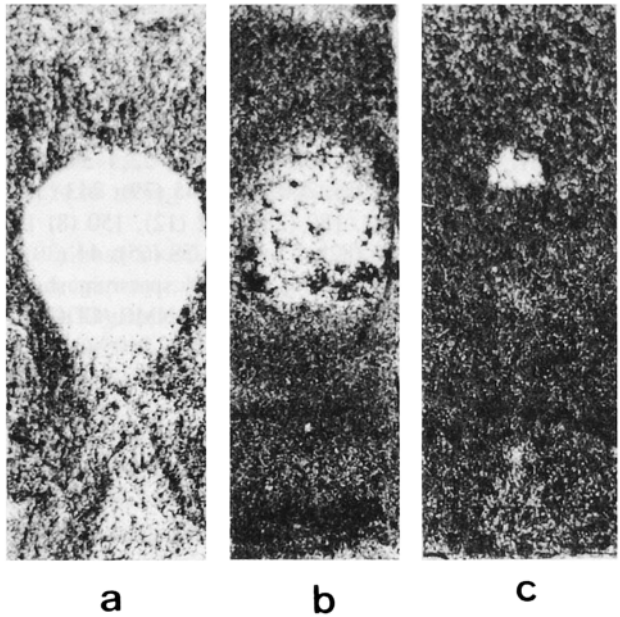

FIG. 2. Fungitoxicity of fatty acids from *Sporothrix flocculosa* culture filtrates on thin layer chromatograms (TLC). After purification with silica gel column, preparative TLC, and HPLC, aliquots (120 μl) from (a) 6-methyl-9-heptadecenoic acid, (b) 4-methyl-7,11-heptadecadienoic acid, and (c) 9-heptadecenoic acid were spotted on silica gel TLC and developed with ethyl acetate–hexane (1:4; v/v). After drying, the plates were sprayed with a conidial suspension of *Cladosporium cucumerinum* and incubated for 48 hr at room temperature.

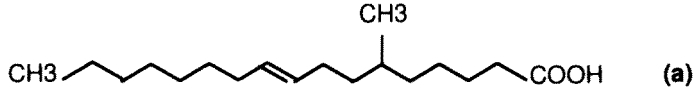

FIG. 3. Structure of the new fatty acid antibiotics: (a) 6-methyl-9-heptadecenoic acid and (b) 9-heptadecenoic acid.

The 6-methyl-9-heptadecenoic acid (Figure 3b) infrared spectrum exhibited strong absorption at 3350, 2680, 1720, 1660, 1420 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) was: $\underline{\delta}$ppm 10.80 (s, 1H), 5.35 (m, 2H), 2.30 (t, 2H), 2.18 (m, 4H), 1.95–2.0 (m, 18H), 0.89 (d, 3H), 0.83 (d, 3H). Cosy NMR (C-H coupling) ($CDCl_3$, 300 MHz) was: δppm 180.2 (s, COOH), 130 (d, CH=CH), 129.56 (d, CH=CH), 45.3 (q, CH_3), 38.3 (s, CH), 22.4–34.0 (t, $12CH_2$), 13.90 (q, CH_3). El-MS: *m/z*: 282 (92), 260 (58), 245 (39), 243 (35), 232 (2), 220 (3), 201 (8), 199 (10), 192 (4), 180 (28), 162 (12), 150 (8) 136 (4) 122 (47) 111 (48), 93 (50), 83 (25), 73 (82), 71 (100), 58 (65), 41 (30).

The 9-heptadecenoic acid (Figure 3b) infrared spectrum showed strong absorption at 3400, 2700, 1710, 1650, 1420 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) was: δppm; 10.85 (s, 1H), 5.38 (m, 2H), 2.30 (t, 2H), 2.05 (m, 4H), 1.6 (m, 2H), 1.2 (m, 18H), 0.85 (t, 3H). Cosy NMR (couplage C—H) ($CDCl_3$, 300 MHz) was: δppm 180.26 (s, COOH), 129.85 (d, CH=CH), 129.56 (d, CH=CH), 22.5–33.9 (t, $13CH_2$), 13.91 (q, CH_3). El-MS: *m/z* 268 (85), 246 (70), 219 (35), 219 (3), 194 (5), 152 (4), 138 (5), 111 (25), 83 (32), 55 (92), 41 (82), 28 (25).

The number of double bonds was confirmed by catalytic hydrogenation using Pt/H_2 and by ^1H and ^{13}C NMR based on integration of ethynilic protons, which indicated the presence of one double bond in both compounds. Ozonolysis of 6-methyl-9-heptadecenoic acid gave a mixture of a monoacid that was identified as octanoic acid by comparison of its analytical data (^1H and ^{13}C NMR) with a commercial product (Aldrich, Milwaukee, Wisconsin), and a decadioic acid branch for which the number of carbons was determined by ^{13}C NMR. Ozonolysis of 9-heptadecenoic acid gave a mixture of two compounds that were identified as octanoic and azelaic acids by comparison of their analytical data with commercial products (Aldrich). These analytical and transformation techniques indicated the presence of a unique double bond between carbons 9 and 10 in the two compounds described. The saturated pyrrolidide derivative of 6-methyl-9-heptadecenoic acid showed a minimum between the fragment $[M\text{-}C_{11}H_{23}]^+$ (molecular ion 182) and the fragment $[M\text{-}C_{13}H_{27}]^+$ (molecular ion 154). This minimum had a molecular ion of 168 corresponding to the fragment $[M\text{-}C_{10}H_{21}]^+$ minus $[CH\text{—}CH_3]$, which confirmed the position of the methyl branch on carbon 6.

It was thus possible to isolate and purify three active compounds from the culture filtrates of *S. flocculosa*, two of which are reported for the first time. In a previous study, Choudhury et al. (1994) had isolated and identified two molecules with antimicrobial activity from *S. flocculosa* and *S. rugulosa*. They were both observed here, but the 4-methyl-7,11-heptadecadienal, which rapidly oxidizes into 4-methyl-7,11-heptadecadienoic acid could only be detected in trace amounts because of its instability at room conditions. The latter compound (4-methyl-7,11-heptadecadienoic acid) was determined to be active to varying

degrees against a number of fungi based on an agar diffusion assay (Choudhury et al., 1994). However, reliability of this bioassay may be variable with apolar compounds such as fatty acids, which do not diffuse readily in agar. In addition, such an assay is not amenable to rapid screening. By a more discriminating bioassay directly on TLC, it was possible to identify two additional fatty acids with antibiotic activity, 9-heptadecenoic acid and 6-methyl-9-heptadecenoic acid, which appear to be more active than 4-methyl-7,11-heptadecenoic acid (Figure 3).

Previous ultrastructural studies had shown that cellular alterations caused in the target fungi were similar to those caused by the organism (Hajlaoui et al., 1992, 1994; Hajlaoui and Bélanger, 1993). Indeed, treatment of target fungi with the fungus or with extracts from its culture filtrates induced similar reactions, such as plasmalemma retraction and the leakage of cytoplasmic content, indicating an alteration of physiological properties of the cytoplasmic membrane (Hajlaoui et al., 1994). However, the molecular mechanism of action of fatty acids with antimicrobial activity has been little studied. It has been suggested that the fluidity of the cell membrane can be disturbed maximally by lipophilic compounds of particular chain lengths, unsaturation, or with particular hydrophilic functional groups (Kabara et al., 1977; Ko et al., 1994). In addition, alteration of the fluidity of the cell membrane by fatty acids was shown to be responsible for the inhibition of some membranous proteins such as α-mannosyl transferase (Watson and Rose, 1980), valyl-tRNA synthetase (Black, 1985) or (1,3)-β-glucan synthase (Ko et al., 1994).

The antimicrobial activity of certain fatty acids has been demonstrated in several studies (Bayliss, 1936; Nieman, 1954; Conley and Kabara, 1973; Kabara et al., 1972, 1977). For instance, some saturated and unsaturated fatty acids inhibited some bacteria such as *Pseudomonas aeruginisa*, *Streptococcus* (group A), and *Staphyloccocus aureus;* yeast fungi such as *Candida albicans* and *Saccharomyces cerevisiae* (Conley and Kabara, 1973, Kabara et al., 1972, 1977); and algae (Ikawa et al., 1984). These studies also reported on structure–function relationships and showed that the optimum antimicrobial activity for even-carbon-numbered aliphatic acids was found in C_{12} saturated fatty acids and C_{18} mono- and diunsaturated fatty acids (Bayliss, 1936; Nieman, 1964; Conley and Kabara, 1973). In addition, it was emphasized that *cis* unsaturated isomers were active while the *trans* isomers were always less active or inactive when compared to the more common unsaturation form (Kabara, 1987).

From an ecological point of view, our findings of antibiotic production by *S. flocculosa* are consequent with its description as an epiphyte. In fact, it is known that this biocontrol agent has a restricted ecological niche in the phylloplan (Traquair et al., 1988), and it would appear plausible that it is protected by antibiotics since it is neither a good competitor nor a parasite (Hajlaoui et al., 1992). In addition to the new antibiotics reported here and the one previously

described by Choudhury et al. (1994), other rare and unusual molecules have been isolated from the organism, the roles of which are still unknown (Choudhury et al., 1995).

The role of antifungal molecules in disease suppression has been shown to be of considerable importance, and the screening of mycelial culture from some biological control agents has led to the discovery of a great number of metabolites with antimicrobial activity (Fravel, 1988). It is evident from this work that *Sporothrix flocculosa*, a biocontrol agent against powdery mildew fungi, produces several antibiotics that account for its rapid and strong activity on target fungi, as demonstrated previously (Jarvis et al., 1989; Hajlaoui and Bélanger, 1991, 1993; Hajlaoui et al., 1994). In this context, screening of *S. flocculosa* isolates for increased production of these molecules should help in selection of strains with superior biocontrol properties.

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EFFICACY OF NONPHEROMONE COMMUNICATION DISRUPTANTS OF CODLING MOTH (*Cydia pomonella*): EFFECT OF PHEROMONE ISOMERS AND OF DISTANCE BETWEEN CALLING FEMALES AND DISPENSERS

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Abstract—Field tests comparing the ability of codlemone, (*E*,*E*)-8,10-dodecadien-1-ol, and isomers of codlemone to disrupt pheromonal communication of codling moth were carried out. In a pear orchard, four nonisomerizing, gray septa dispensers were placed in the upper canopy of each tree containing a trap baited with 10 virgin female codling moths. The dispensers were at trap height and 70 cm from the edge of each trap. Trap catches of released male codling moths in three test areas were compared simultaneously when trees in each of the test areas contained unbaited dispensers, dispensers with 1 mg of codlemone containing 1% isomers, and dispensers with 1 mg of a test communication disruptant. When the test disruptant was an equilibrium mixture of codlemone and its isomers (61% codlemone, 39% isomers), the percent communication disruption was 86.8% compared to 68.7% for codlemone ($P < 0.001$). When the disruptant was (*E*,*Z*)-8,10-dodecadien-1-ol (94% *EZ*, 3% *EE*), the percentage disruption was 86.4% compared to 62.7% for codlemone ($P < 0.002$). These results show that the previously reported superior disruptant potency (relative to codlemone) of compositions containing codlemone with a high percentage of isomers was not a result of the proximity of the dispensers to the traps. The percent disruption of compositions of codlemone with 10 and 20% isomers was also determined. A plot of percentage disruption versus logarithm of percentage of nonpheromone isomers in the mixture from 1% to 97% gave a straight line with $R^2 = 0.93$.

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Key Words—Codling moth, *Cydia pomonella*, sex pheromone, codlemone, (*E*,*E*)-8,10-dodecadien-1-ol, sex pheromone isomers, 8,10-dodecadien-1-ol isomers, (*E*,*Z*)-8,10-dodecadien-1-ol, mating disruption, communication disruption.

INTRODUCTION

Previously, we reported (McDonough et al., 1994) that the (*E*,*Z*)-8,10- and (*Z*,*E*)-8,10- isomers of codlemone, (*E*,*E*)-8,10-dodecadien-1-ol, and the equilibrium mixture of isomers of codlemone (61% *EE*, 20% *EZ*, 14% *ZE*, and 5% *ZZ*) were more potent communication disruptants than codlemone itself. These results were unexpected, because the sex pheromone of the codling moth is the single component, (*E*,*E*)-8,10-dodecadien-1-ol (Roelofs et al., 1971; McDonough et al., 1995), and based on reports of other insects, it was expected that the natural pheromone would be a more potent communication disruptant than nonpheromone compounds.

In earlier studies of a nonpheromone communication disruptant of *Trichoplusia ni* (cabbage looper), Tumlinson et al. (1972) reported inhibition of trap catch when the disruptant was mixed with the pheromone at levels as low as 0.1% of the pheromone. Subsequently, Kaae et al. (1974) and McLaughlin et al. (1974) tested the inhibitor in widely spaced evaporators. When the amount of the inhibitor was as high as 10 times the amount of pheromone that elicited 84% communication disruption, no disruption was obtained. McLaughlin et al. (1974) proposed that the inhibitor was perceived by a sensory neuron separate from that for the pheromone and that the insect could resolve the inhibitor and the pheromone signal, and then, through a more central integrative process, become habituated to the inhibitor while remaining responsive to the pheromone. More recently, the proposal of McLaughlin et al. (1974) gained support from the studies of Liu and Haynes (1992, 1993) on the cabbage looper and Rumbo et al. (1993) on the light-brown apple moth.

It is not known if the isomers of the codling moth pheromone are detected by sensory neurons separate from those for the pheromone. If they are, and the proposal of McLaughlin et al. (1974) were applicable to the codling moth, then a possible reason for our different results (McDonough et al., 1994) was that the disruptant dispensers were too close to the pheromone source (about 10 cm from the trap edge). Part of the isomer concentration may have mixed thoroughly enough with the much lower concentration of pheromone emitted by the females that the mixture was perceived as one (Liu and Haynes, 1992). Furthermore, if false trail following is an important mechanism for communication disruption, then the pheromone might gain in relative effectiveness as the distance between the traps and the disruptants were increased. For these reasons, this study was undertaken to retest the isomers with a distance of 70 cm between the disruptants

and the edge of the female baited traps, and if the previous results were confirmed, to determine the disruptant potency over a range of isomer concentrations.

METHODS AND MATERIALS

Chemicals. (*E*,*E*)-8,10-Dodecadien-1-ol (Bedoukian Research Inc., Danbury, Connecticut) was recrystallized from pentane and was 99.0% isomerically pure by capillary gas chromatography. (*E*,*Z*)-8,10-Dodecadien-1-ol (94% *EZ*, 3% *EE*, 1% *ZE*, 2% *ZZ*) was synthesized by the method of McDonough and Smithhisler (1989) for the synthesis of (*E*,*Z*)-10,12-tetradecadien-1-ol except that 1,8-octanediol was used in place of 1,10-decanediol. The equilibrium mixture of isomers of 8,10-dodecadien-1-ol was prepared as previously reported (McDonough et al., 1993). The isomeric content was 61% *EE*, 20% *EZ*, 14% *ZE*, and 5% *ZZ*. The compositions containing 10% and 20% isomers were prepared by mixing the appropriate quantities of the compositions containing 1% and 39% isomers.

Disruptant Dispensers. Gray elastomeric septa (size 1F, formulation #1888, The West Co., Lionville, Pennsylvania) were used to dispense disruptant compositions because they minimize isomerization of conjugated dienes (Brown and McDonough, 1986). The dose was 1 mg/septum for all compositions. Each composition was added to the cup of the dispenser in 200 μl of dichloromethane.

Traps. The trap was a modified Pherocon 1 (Trécé Corp., Salinas, California), and was identical to that previously described and pictured (McDonough et al., 1994) except that the wires for holding the disruptant dispensers were deleted. Each trap was baited with 10 females in wire cages.

Insects. Pupae were obtained from a laboratory colony reared on artificial diet (Howell, 1972; Toba and Howell, 1991). Individual pupae were placed in 15 × 50-mm vials and maintained at 20°C and 68% relative humidity on a 10:14-hr dark–light cycle. After emergence, the adults in their vials were sexed under a magnifying glass. In all experiments, both male and female codling moths were used two to four days after eclosion.

Field Test Area. A commercial pear orchard was used for the tests (Figure 1). The intrarow distance between adjacent trees was 6.1 m and the separation between rows was 6.1 m. Males were released in the stipple-designated trees of Figure 1. Each stipple-designated tree was surrounded by four black-designated trees, each of which contained a trap baited with 10 female codling moths. There were six groups of these trees (Figure 1) and two groups were used for each test composition. The foliar dimensions of the trees were about 4.6 m high and about 5.8 m in diameter. To facilitate placement and removal of the traps, a 1.9-cm-OD steel pipe was driven into the ground next to each tree trunk. The

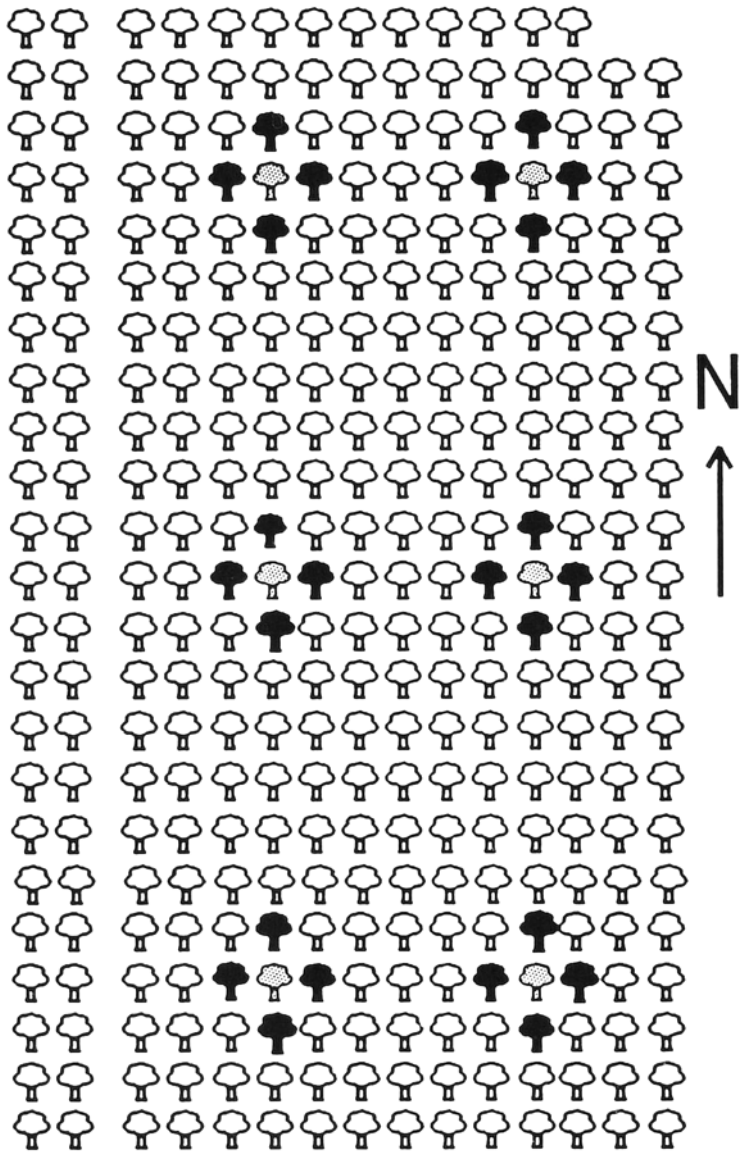

Fig. 1. Map of the section of the pear orchard used for communication disruption tests. Males were released in the stipple-designated trees. Female baited traps and disruptant dispensers were placed in the black-designated trees.

steel pipe extended to a height of about 4 m. A metal shelf bracket was attached to the top of the pipe by means of a steel hose clamp. A wire hook was attached to the trap and one to the shelf bracket, and the trap was hooked to the shelf bracket hook by a wire hook on the end of a long pole. The traps were placed at a height of about 3.5 m. There were four disruptant dispensers on each tree containing a female-baited trap. The dispensers were wired to a limb in the four cardinal directions at trap height, 80 cm from the center of the trap and about 70 cm from the edge of the trap.

Test Protocol. The test data of each week consisted of the sum of two three-day test periods: Tuesday–Thursday and Friday–Sunday. Since a commercial orchard was used, feral populations of male codling moths were expected to be low and unevenly distributed. Therefore, dyed males were released to provide sufficient numbers and even distribution. The dyes were nontoxic (Moffitt and Albano, 1972) and were from Day-Glo Color Corp., NALCO Chemical Co., Cleveland, Ohio. Male and female moths were put out on Tuesdays and Fridays at midday and were removed when trap catches were counted at approximately the same time on Fridays and Mondays. Males and females in their vials were chilled in a refrigerator to facilitate their transfer. Ten females were placed in each of the 24 15-mesh aluminum wire cages (240 females/release or 480 females/week), and 30 males were placed in each of four cylindrical cartons [8.5 cm diameter × 9 cm high, internally coated with a dye (Moffitt and Albano, 1972)] at each release tree (720 males/release or 1440 males/week). The males were held in their cartons for an hour. Then the cartons were placed in galvanized wire screen holders (2-mesh and 11 cm diameter × 13 cm high) with a galvanized wire-screen lid to prevent bird predation, and the lids of the cartons were removed. The cartons were placed at a height of about 1.5 m. On the days when traps were removed, the number of males remaining in the cartons and the number of dead females in the cages were counted. In these tests, insect mortality was always low. For the season, 4.3% of the caged females were dead when the traps were removed from the trees, and 2.3% of the males failed to leave their cartons. The recapture rate in the control was usually 40–60%, with a high of about 70%. When the recapture rate was below 30%, the experiment was repeated. Feral males were rarely caught, and then only one or two per test, and were not included in the counts. Each east-west couplet of tree groups (Figure 1) (one male release tree and four trees with female baited traps per group) was used for a different test composition: the control "composition" was always blank, another was always codlemone, and the third was the isomeric mixture being evaluated. After each week the positions of the test compositions were changed, so that after three weeks each composition had occupied each position for one week. Each complete test required three weeks. The isomeric disruptant mixtures in the four tests were as follows: (1) the equilibrium mixture

of codlemone and isomers (39%), (2) codlemone and isomers (20%), (3) codlemone and isomers (10%), and (4) (*E,Z*)-8,10-dodecadien-1-ol (94% *EZ*, 3% *EE*, 2% *ZZ*, 1% *ZE*).

Calculations and Statistical Analysis. The percent disruption was calculated by determining the catch in a test area as a percent of the catch in the control area and subtracting that value from 100. Because different colored males were used for each test area, the data could be analyzed either in terms of total catch or catch of the males of the color for that area. The two analyses yielded equivalent results, and the results reported are for total catch. Data were analyzed with the Statistical Analysis System (SAS) general linear models procedure (SAS Institute, 1985) to test for differences between codlemone (1% isomers) and one of the test materials.

Linear Regression of Percentage Disruption Versus Logarithm of Percentage of Nonpheromone Isomers. For this calculation the four values for percentage disruption by codlemone (1% isomers) were averaged. The values for the other four compositions were normalized to this average value. The values plotted were 1, 10, 20, 39, and 97% nonpheromone isomers. The 97% value was obtained from (*E,Z*)-8,10-dodecadien-1-ol, which had different proportions of the nonpheromone isomers than the other four compositions. This was justified because in our previous study (McDonough et al., 1994), the *EZ* and *ZE* isomers elicited equal percentages of disruption.

RESULTS AND DISCUSSION

The results of the four major tests are summarized in Table 1. In test 1 the equilibrium mixture of isomers of 8,10-dodecadien-1-ol (39% non-*EE* isomers) gave a higher percentage of communication disruption than codlemone (1% non-*EE* isomers) and the difference was highly significant ($P < 0.001$). In test 2 (*E,Z*)-8,10-dodecadien-1-ol gave a higher percentage of communication disruption than codlemone, and again the difference was highly significant ($P < 0.002$). These tests support our earlier report (McDonough et al., 1994) and establish that the superiority of the isomers as communication disruptants was not dependent on the proximity of the disruptants to the calling females, as previously described.

In the case of the pea moth (*Cydia nigricana*), superior disruption of communication was also found when isomers of the pheromone, (*E,E*)-8,10-dodecadien-1-ol acetate, formed (Bengtsson et al., 1994). The authors reported that males moved out of the field as the isomers formed. When mixed with the pheromone as an attractant, the isomers of the pea moth pheromone were potent inhibitors (Witzgall et al., 1993), whereas, when the isomers of the codling moth pheromone were mixed with the pheromone as an attractant, the inhibitory

TABLE 1. COMPARISON OF PERCENT COMMUNICATION DISRUPTION OF CODLEMONE (*E,E*)-8,10-DODECADIEN-1-OL (99%; 1% ISOMERS) WITH FOUR OTHER COMPOSITIONS HAVING HIGHER ISOMER CONTENT

Test	Composition	Mean % disruption (± SEM)	*P*
1	Codlemone (99%; 1% isomers)	68.70 ± 13.01	<0.001
	Codlemone (61%; 39% isomers)	86.75 ± 2.2	
2	Codlemone (99%; 1% isomers)	62.65 ± 6.03	<0.002
	(*E,Z*)-8,10-dodecadien-1-ol (94%; 3% codlemone; 2% *ZZ*, 1% *ZE*)	86.43 ± 2.72	
3	Codlemone (99%; 1% isomers)	62.13 ± 7.41	<0.011
	Codlemone (80%; 20% isomers)	82.92 ± 4.42	
4	Codlemone (99%; 1 isomers)	57.40 ± 19.90	<0.175
	Codlemone (90%; 10% isomers)	68.63 ± 4.71	

effect was moderate and the mixture was still significantly attractive (McDonough et al., 1993; Roelofs et al., 1972). These differences indicate that the disruption mechanisms of the isomers are not necessarily the same for these two species.

Recently, Mayer and Doolittle (1994) reported synthetic sex pheromone analogs that synergized responses of the sex pheromone olfactory specialist neurons of the main sex pheromone components of male cabbage looper (*Trichoplusia ni*) and corn earworm (*Helicoverpa zea*) moths. The synergism in the electrophysiological studies was corroborated by behavioral tests of upwind anemotactic responses in flight tunnel tests. It may be that the isomers of the codling moth sex pheromone also exert their inhibitory effect by interaction with the sex pheromone olfactory specialist neuron. Then the codling moth would not be able to resolve the inhibitor signal from the pheromone signal, even when there was a spatial difference between the pheromone and the inhibitor. In contrast, as pointed out earlier, the ability of the cabbage looper to locate a pheromone source was not disrupted when the pheromone inhibitor was separated from the pheromone, and in that case the inhibitor was perceived by a sensory neuron separate from that for the pheromone (McLaughlin et al., 1974).

Tests 3 and 4 in Table 1 show the percentage of communication disruption for the blends containing 10 and 20% isomers. Again both compositions elicited higher percentages of communication disruption than did codlemone, and the results were highly significant for the 20% composition ($P < 0.011$), but less so for the 10% composition ($P < 0.175$) as might be expected for the lower isomer percentage.

Although the total doses of test disruptants were constant in the four tests,

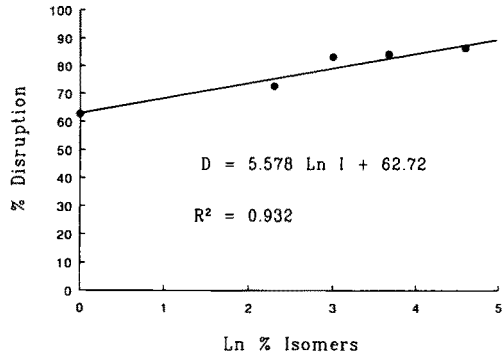

FIG. 2. Relationship between percent communication disruption and natural logarithm of the percentage of nonpheromone isomers from 1 to 97% isomers.

the doses of pheromone and of the isomers in the mixtures varied. Previously we found that a plot of logarithm of dose versus percentage of disruption gave good straight lines for both codlemone and the equilibrium mixture of isomers (McDonough et al., 1994). Therefore, their combination with varying proportions of pheromone and isomers was also expected to give a straight line. A plot of the logarithm of the percentage of isomers versus the percentage of disruption is shown in Figure 2. The correlation, $R^2 = 0.932$, indicates the relationship is linear.

Because of factors such as multiple mating of males, the percentages of communication disruption, as measured by trap catch, may not correlate quantitatively with the percentages of mating disruption. Nevertheless, disruption of communication is the mechanism by which mating disruption is achieved. Because the degree of communication disruption of the isomeric compositions was determined relative to codlemone, it is reasonable to expect that these relative differences will carry over to mating disruption, and therefore the isomers should have practical value in control programs.

Acknowledgments—We thank the Washington State Tree Fruit Research Commission for a grant to partially support this work, Dave Allan for providing the pear orchard for these tests, Tina Johnson and Tyson Miller for technical assistance, and Dr. Gary Richardson, Agricultural Research Service, Fort Collins, Colorado, for advice on statistical analysis of the data.

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EFFECTS OF ORGANIC SOLVENT EXTRACTS FROM HERBAGE ON FEEDING BEHAVIOR IN GOATS

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Abstract—The effects of organic solvent extracts from perennial ryegrass (*Lolium perenne* L. cv. Friend), orchardgrass (*Dactylis glomerata* L. cv. Akimidori) and *Lolium* × *Festuca* hybrid on the hay consumption of goats were measured by simple two-choice feeding assays. Orchardgrass hay treated with methanol extracts (resuspended in water) of perennial ryegrass showed an increased intake by goats compared to untreated hay ($P < 0.01$). The palatability of hay tended to be slightly decreased by spraying it with pentane extract of perennial ryegrass and orchardgrass. Furthermore, extracts of hybrid with pentane inhibited goats from feeding ($P < 0.05$). Methanol extract stimulated hay consumption whereas the pentane extracts did not change or reduce intake.

Key Words—Goat, herbage, organic solvent extracts, feeding stimulants, perennial ryegrass, orchardgrass, hybrid.

INTRODUCTION

Secondary chemicals in food plants are thought to stimulate plant selection in domestic and wild herbivores through olfactory and gustatory senses (Müller-Schwarze, 1991). Arnold (1966) demonstrated that the two senses contributed to the selection of plants by using sensory-impaired sheep. Many researchers have reported on plant chemicals that deter wild and domestic herbivores from feeding on plants (Schwartz et al., 1980; Elliott and Loudon, 1987; Sunnerheim-Sjöberg, 1992; Estell et al., 1994).

However, there is very little information about the presence of stimulative

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chemicals and odors in plants and their effects on domestic herbivores, although use of feeding stimulants to increase intake and improve productivity is promising. Limited information on stimulants, including glucose, butyric acid, and sodium glutamate, was obtained by assaying chemicals already known to be present in herbaceous plants (Arnold et al., 1980; Grovum and Chapman, 1988; Gherardi and Black, 1991). In order to accumulate more information about stimulants, it is necessary to investigate extracts of herbage and their fractions for palatability to domestic herbivores and to then identify specific stimulants.

Organic solvent extracts from palatable and widely used herbage was examined for potential effects on herbage consumption by goats. The extractions were conducted with two organic solvents with different polarities. Behavioral choice-test bioassays using goats were used to test the biological activity of extracts.

METHODS AND MATERIALS

Animals and Management. Six castrated male Siba goats (2–3 years old and weighing 35–55 kg) were used in the experiments. Except during experimental periods, they were maintained on pasture from about 10:00 to 16:00 hr and then fed a small amount of concentrate (main ingredients: barley and soybean) to lure them into their barns each evening. Water, orchardgrass hay, and mineral blocks were provided *ad libitum* in the barn. During experimental periods animals were individually housed and fed in 2-m^2 pens. Throughout the experimental periods, water and mineral blocks were available *ad libitum* and each animal was fed 200 g of hay from 17:00 to 09:00 hr. The hay, which was second cutting orchardgrass in the vegetative growth stage obtained in July 1994 from the National Grassland Research Institute, was chopped in a chaff cutter to about 1.5-cm lengths. The prepared hay also was used in the two-choice tests. Before the experiments, the goats were allowed five days to become adjusted to experimental conditions.

Organic Solvent Extraction. Perennial ryegrass, orchardgrass, and hybrid grass in the vegetative growth stage were collected from grasslands in October 1994, and then cut to about 1-cm lengths with scissors. The cut leaves (260 g) were added to 500 ml of *n*-pentane and blended with a mixer at 10,000 rpm for 5 min while being cooled in an ice bath. The solution was filtered, concentrated to 100 ml in a draft chamber, and then stored at −20°C.

Methanol extract of perennial ryegrass was obtained as described above and stored at −20°C. Preliminary experiments demonstrated that methanol alone inhibited feeding by goats immediately after spraying it on hay. Therefore, 200 ml of distilled water was added to the methanol extract, methanol was removed with a rotary evaporator at 60°C for about 1 hr under vacuum, and the aqueous portion was stored at −40°C. Before the bioassays, the aqueous solution was

adjusted to 100 ml with distilled water. The pentane and methanol solvents used in these experiments were of analytical reagent grade.

Two-Choice Test. Tests were conducted using pentane extracts from perennial ryegrass, orchardgrass, hybrid grass and methanol extracts from perennial ryegrass over a four-day period. Each pen contained two troughs placed 50 cm apart, with one containing 600 g of the hay cut into 1.5-cm lengths and sprayed with 50 ml of a methanolic aqueous extract solution (130 g equivalent of perennial ryegrass). The other trough contained control hay sprayed with 50 ml of distilled water. The prepared fodders were fed to goats immediately. The amount of intake from each trough over a 2-hr period was measured for each goat. The methanolic aqueous extract tests were repeated twice from about 10:00 to 12:00 hr and from 14:00 to 16:00 hr on the same day with the exception of the positions of the troughs (left vs. right), which were switched each time. The significance of differences between the total intake volumes over the 4-hr period was examined via Student's paired t test. Each of the three pentane extracts was tested in the same manner, with pentane used as a control. The troughs were cleaned before any new treatments were sprayed to ensure that no residual substances remained.

RESULTS AND DISCUSSION

It is known that livestock preference for different plant species, plant varieties, and plants at different stages of growth is variable (Garner, 1963; Bryant et al., 1976; Evans and Potter, 1984; Colebrook et al., 1990). However, it is unclear whether chemical constituents are responsible for these differences. This study was designed to extract feeding stimulants from herbage given to domestic herbivores. Arnold et al. (1980) and Gherardi and Black (1991) reported that a bioassay using hay treated and untreated with chemical compounds was suitable to evaluate the effects on feeding by sheep. Their methods were adopted in these experiments, but modified to use organic solvent extracts from herbage with goats as the experimental animals. This choice test enabled us to assess the effects of specific extracts on forage preference with other factors affecting herbage preference excluded (i.e., physical factors, insoluble chemicals etc.).

Because herbage odor is thought to play an important role in stimulating livestock feeding (Müller-Schwarze, 1991), the herbages were first extracted with pentane, which should extract most of the volatile chemicals in the herbage (Blanch et al., 1991). However, pentane extracts of perennial ryegrass and orchardgrass did not stimulate hay consumption by goats, rather it tended to deter intake. The hybrid extract significantly inhibited feeding by goats (Figure 1).

These results suggested that chemicals extracted by pentane inhibited feed-

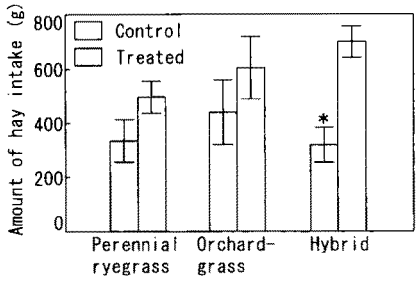

FIG. 1. Effect of *n*-pentane extracts from three types of herbage on hay intake by goats ($N = 6$) in two-choice tests. Asterisk and vertical bars denote significance ($P < 0.05$) and standard errors, respectively.

ing by goats. However, pentane extracts included not only volatile chemicals but also nonvolatile compounds that may deter consumption. Furthermore, pentane extracts may not reproduce the exact odor of the herbage due to the incomplete quantitative extraction and/or release of the volatile chemicals contained in the herbage. When hay was sprayed with pentane extracts, it lost its natural odor due to a blending with the odors from the pentane extracts. Goats might perceive sprayed hay as unfamiliar food with an odor different from that of hay or herbage. Some authors reported herbivores were unwilling to eat a food with which they were not familiar (Arnold and Maller, 1977; Hutson and van Mourik, 1981; Chapple et al., 1987; Burritt and Provenza, 1991). Therefore, the low preference goats have for hay sprayed with pentane extracts may be due to a lack of familiarity with the chemicals added to the hay from the extracts rather than to a congenital dislike of the chemicals in the extracts. Based on results obtained in these experiments, we concluded that no specific volatile chemicals with an intensely stimulative activity were present in the pentane extracts.

Aqueous solutions obtained from methanol extracts increased hay intake by goats even though the goats were unfamiliar with hay sprayed with the extract, suggesting that the extract contained strongly stimulative chemicals (Figure 2). The methanol extract likely contained more polar chemicals than the pentane extract. Because most of the methanol was removed from the extract before it was sprayed on hay, the solution probably contained less volatile chemicals than the pentane extract. Therefore, it seems that gustatory chemical substances that were detected by goats stimulated food intake more than odors. However, during this process chemicals in the methanol extracts may also be modified by heating, and it is possible that modified chemicals not present in perennial ryegrass are stimulants. Glucose, monosodium glutamate, and butyric acid were reported to stimulate drinking and feeding behavior of cattle and sheep

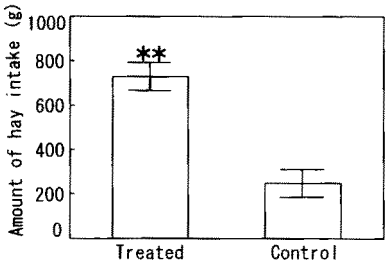

FIG. 2. Effect of methanol extract from perennial ryegrass on hay intake by goats (N = 6) in two-choice tests. Asterisks and vertical bars denote significance ($P < 0.01$) and standard errors.

(Arnold et al., 1980; Grovum and Chapman, 1988; Gherardi and Black, 1991). These substances would likely be extracted with methanol from plant tissues. Thus, the presence of these compounds might partially explain the stimulative effect of the methanol extract, but other chemical stimulants may also be present.

This study was a preliminary one aimed at elucidating what kinds of chemical constituents in herbage are responsible for preferences among domestic herbivores. Isolation and identification of the active compound(s) in the methanol extract of perennial ryegrass is currently under way. As for the pentane extracts, it will first be necessary to test the activity at higher extract concentrations than those used in these experiments because the weight of forage for extraction in this study may be too low to permit goats to detect palatable chemicals in the pentane extract. Furthermore, we should determine whether the presence of unpalatable chemicals in the extracts or unfamiliarity with the hay sprayed with them plays an important role in the low preference of goats for pentane extracts.

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PHEROMONE SPECIFICITY IN *Eriocrania semipurpurella* (Stephens) AND *E. sangii* (Wood) (LEPIDOPTERA: ERIOCRANIIDAE) BASED ON CHIRALITY OF SEMIOCHEMICALS[1]

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Abstract—The fifth abdominal segment of female *Eriocrania semipurpurella* (Stephens) and *E. sangii* (Wood) contains a pair of exocrine glands. Hexane extracts of this segment were prepared from both species and analyzed by gas chromatography with simultaneous flame ionization and electroantennographic detection (EAD). For both species, the EAD active peaks were identified as nonan-2-one, (*Z*)-6-nonen-2-one, and (*Z*)-6-nonen-2-ol by means of mass spectrometry and comparison of retention indices with those of synthetic standards. Enantiomeric separation of chiral alcohols from the female extracts was achieved by gas chromatographic analysis on a cyclodextrin column. In *E. semipurpurella*, a mixture of (2*S*,6*Z*)-nonen-2-ol and (2*R*,6*Z*)-nonen-2-ol

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[1]Dedicated to Prof. H. J. Bestmann on the occasion of his 70th birthday.

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(2 : 1) was found, whereas in *E. sangii* (2*S*,6*Z*)-nonen-2-ol was the predominant enantiomer and only traces of the *R* enantiomer were indicated by the antennal response. In field tests, a blend of the three compounds was not attractive to conspecific males. A subtractive assay showed that the alcohol in various enantiomeric mixtures was the only attractive compound, whereas addition of (*Z*)-6-nonen-2-one to the alcohol completely inhibited the attraction of both species. A trapping experiment including a wide range of ratios between the *R* and *S* enantiomers showed that baits containing 95–100% of the *S* enantiomer were attractive to male *E. sangii*, whereas males of *E. semipurpurella* were attracted to all tested ratios of the enantiomers. However, the response profiles of male *E. semipurpurella* differed between populations from southern Sweden, south Finland, and the Kola Peninsula in Russia. In south Sweden males were maximally attracted to a racemic mixture of the alcohols. At the Kola Peninsula *E. semipurpurella* was attracted to baits containing 95–100% of the *R* enantiomer. In south Finland all tested ratios between 0 and 100% *R* enantiomer trapped *E. semipurpurella*, but the trap catches appeared to be bimodally distributed with peaks around 15 and 70% *R* enantiomer. The trapping results suggest the existence of pheromone races or sibling species among the specimens identified as *E. semipurpurella*.

Key Words—*Eriocrania semipurpurella*, *Eriocrania sangii*, Eriocraniidae, Lepidoptera, sex pheromone, GC-EAD, chiral gas chromatography, mass spectrometry, synthesis, field trapping, nonan-2-one, (*Z*)-6-nonen-2-one, (2*S*,6*Z*)-nonen-2-ol, (2*R*,6*Z*)-nonen-2-ol.

INTRODUCTION

Sex pheromones identified from the higher moths, the so-called Ditrysia, are remarkably similar in chemical structure. The pheromone components identified from more than 400 species of higher Lepidoptera generally consist of aliphatic fatty acid derivatives with 10–20 carbon atoms in the carbon skeleton (Arn et al., 1992). Recent studies on *Eriocrania cicatricella* (Zhu et al., 1995) and *Stigmella malella* (Tóth et al., 1995) showed that these nonditrysian moths possess pheromones that differ in chemical structure from those of ditrysian Lepidoptera, but are very similar to pheromones of caddisflies with respect to both chemistry and site of pheromone production (Löfstedt et al., 1994; Zhu et al., 1995). However, data on pheromones of the first evolutionary lineages of the order Lepidoptera are still very scanty, and more identifications of pheromones from species in such lineages are needed for understanding the evolution of the lepidopteran pheromone communication systems.

The present study of two eriocraniid moths was aimed at the identification of the pheromones of two closely related species, *E. semipurpurella* (Stephens) and *E. sangii* (Wood). The study was promoted by a forthcoming taxonomic revision of the family Eriocraniidae (O. Karsholt, personal communication), which shows a large morphological variation in the two species and reveals

problems in their identification. Additionally, these species recently became the target objects for a study of leaf-miner responses to aerial emissions of nickel–copper smelters (Koricheva and Haukioja, 1992; Kozlov and Haukioja, 1993), which created an urgent need for synthetic blends to monitor their population densities.

METHODS AND MATERIALS

Insects. Moths of *E. semipurpurella* and *E. sangii* were collected as adults from young birch forests on May 1–6, 1992 (Turku, Finland), April 21–30, 1993, and April 24–28, 1994 (Lund, Sweden). Adults were sexed immediately after capture, kept in plastic vials containing moist filter paper, and transported to the laboratory in Lund for electrophysiological tests and chemical analyses.

Since identification of these species sometimes is problematic, it should be mentioned that the key character we used to discriminate the two species was the form of the terminal segment of the long, folded maxillary palp; specimens with a pointed apex of this segment were attributed to *E. semipurpurella*, whereas the presence of a clear bifurcation indicated *E. sangii* (O. Karsholt, personal communication).

Preparation of Extracts. Extracts of the fifth abdominal segment from adult females were used for gas chromatography with electroantennographic detection (GC-EAD). The insects were anesthetized by cooling them to −20°C before dissection. The dissected segment of an individual female was extracted for at least 30 min in a pointed tube containing 20 μl redistilled hexane. These extracts were subsequently used for both electrophysiological tests and chemical identification.

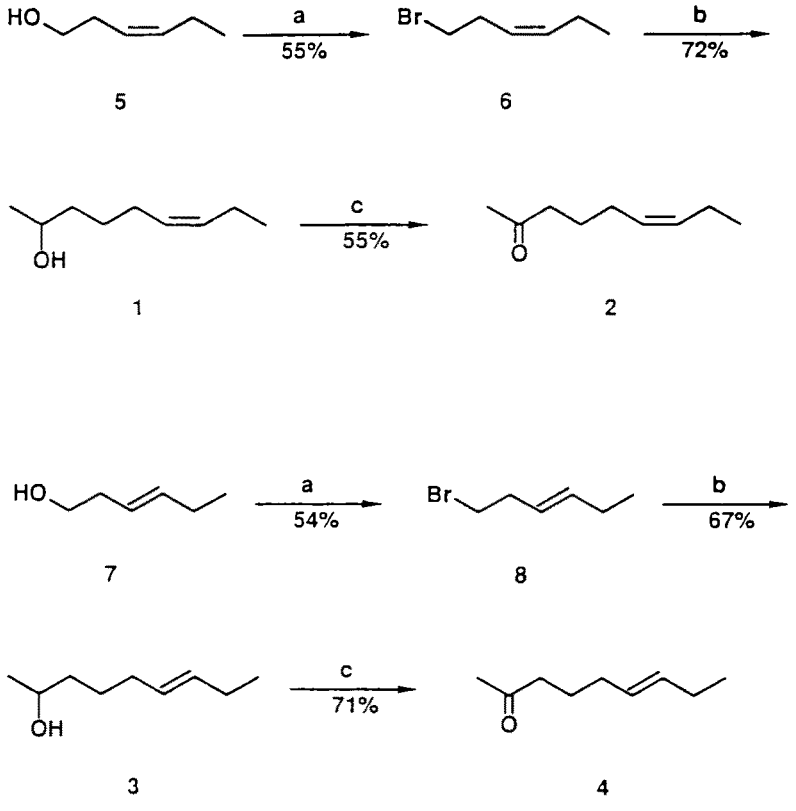
Chemical Analyses. To examine male antennal sensitivity to extracts of females of the two species, the tip of the male antenna was cut off and the antenna was mounted between two pipet electrodes containing Beadle-Ephrussi Ringer, and grounded via an Ag–AgCl wire. The tip electrode was connected to a high-impedance DC amplifier with automatic baseline drift compensation. GC-EAD analyses of extracts of female fifth abdominal segments of the two species were conducted on a Hewlett Packard 5830 GC equipped with either of two different columns, a DB-WAX column (30 m × 0.25 mm ID, J & W Scientific, Folsom, California) or a cyclodextrin column (25 m × 0.25 mm ID) coated with a mixture of 6-*O*-methyl-2,3-di-*O*-pentyl-γ-cyclodextrin and OV-1701 in a ratio of 1:4 (Prof. W. A. König, Institute of Organic Chemistry, University of Hamburg, Hamburg, Germany). An effluent split allowed simultaneous flame ionization (FID) and electroantennographic detection (EAD) of the separated pheromone components. Hydrogen was used as carrier gas, and the effluent split ratio was approximately 1:1. Samples were injected splitless.

The split valve was opened 1 min after injection. The injector temperature was kept at 250°C and the column temperature was maintained at 40°C for 3 min following the injection on the DB-WAX column and then linearly increased to 230°C at a rate of 5°C/min. When using the cyclodextrin column, the injector temperature was kept at 150°C and the column temperature was maintained at 60°C for 3 min following the injection and then linearly increased to 160°C at a rate of 5°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. Either 5 μl of extracts containing 5–10 female equivalents or 5 μl of an individual female abdominal segment extract were injected into the columns for the analyses. Gas chromatographic analyses of the geometric isomers were performed using a fused-silica capillary column (50 m × 0.32 mm ID) coated with Free Fatty Acid Phase (FFAP), programmed from 60 to 220°C at a rate of 5°C/min.

Mass spectra of EAD active compounds were recorded first on a Hewlett Packard 5970 GC-MSD equipped with a DB-WAX column, programmed from 40 to 230°C at a rate of 5°C/min and, second, with a double-focusing instrument (VG 70-250 SE; Vacuum Generators, Manchester, England) at 70 eV by using a fused-silica capillary (50 m × 0.32 mm ID) coated with FFAP, programmed from 60 to 220°C at a rate of 5°C/min. Microhydrogenation of natural products was carried out according to Huwyler (1973). Enantiomeric separation was achieved using a cyclodextrin column operated under the following conditions: 12 min at 60°C, then linearly increased to 120°C at a rate of 3°C/min. NMR spectra (400.1 MHz for ^{1}H and 100.6 for ^{13}C) of synthetic samples were run on a Bruker AMX 400; δ TMS = 0, δ $CDCl_3$ = 7.26 (s = singlet, bs = broad singlet, d = doublet, t = triplet, qu = quintet, m = multiplet, dtt = doublet of a triplet of a triplet, dbq = doublet of a broad quartet, dbqu = doublet of a broad quintet); attribution of ^{13}C signals was made according to DEPT spectroscopy (CH_3 and CH = +; CH_2 = −; quart C = 0).

Chemical Syntheses. Our straightforward syntheses of the target compounds are shown in Scheme 1. Commercially available 3-hexen-1-ols were converted into the corresponding bromides according to Wijnberg et al. (1981). Grignard reaction with epoxypropane (Johnston and Slessor, 1979) afforded 6-nonen-2-ols, which were subsequently oxidized to the ketones following the method of Herscovici et al. (1982). Optically active (*Z*)-6-nonen-2-ols were produced from (*Z*)-3-hexen-1-ol and optically active epoxypropanes.

(Z)-1-Bromo-3-hexene (6). A solution of 16.0 g bromine (0.11 mol) in 20 ml dichloromethane was added at −10°C to a stirred solution of 26.2 g (0.11 mol) of triphenylphosphane in 120 ml dichloromethane. Subsequently at the same temperature, 10.0 g (0.10 mol) of commercially available (*Z*)-3-hexen-1-ol (Aldrich) dissolved in 40 ml dichloromethane was added dropwise. After 30 min the mixture was poured on 500 ml water, the organic layer was separated,



a) PPh_3Br_2/ CH_2Cl_2 ; b) 1.Mg,CuI/ THF 2.methyloxirane ;
c) PDC, MS4 Å

SCHEME 1.

and the aqueous phase was extracted twice with 500 ml of dichloromethane. The combined organic phase was washed twice with saturated sodium bicarbonate, dried over magnesium sulfate, and concentrated under reduced pressure. The crude product was distilled in vacuo yielding 9.0 g (55%) of **6**, bp_{12}: 60–61°C.

^{1}H NMR (400.1 MHz, $CDCl_3$): δ = 5.59 (dtt, 1H, J = 10.8 Hz, J = 7.2 Hz, J = 1.5 Hz), 5.37 (dtt, 1H, J = 10.8 Hz, J = 7.2 Hz, J = 1.5 Hz), 3.37

(t, 2H, $J_{1,2}$ = 7.2 Hz, H-1), 2.62 (dbq, 2H, $J_{2,3}$ = 7.2 Hz, $J_{2,4}$ = 1.5 Hz, H-2), 2.06 dbqu, 2H, $J_{5,4}$ = $J_{5,6}$ = 7.2 Hz, H-5), 0.98 (t, 3H, $J_{6,5}$ = 7.6 Hz, H-6). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 134.75 (+), 125.20 (+), 32.85 (−), 30.75 (−), 20.74 (−), 14.16 (+). MS; *m/z*, 39 (50), 41 (100), 53 (16), 55 (94), 67 (31), 69 (13), 82 (14), 83 (50), 162 (11, M^+), 164 (11, M^+).

rac.(Z)-6-Nonen-2-ol (1). From 2.5 g (15.3 mmol) of **6,** dissolved in 25 ml tetrahydrofurane and 0.38 g (15.6 mmol) magnesium turnings, a Grignard reagent was prepared. After addition of 0.24 g (1.3 mmol) copper(I) iodide, the mixture was stirred for 15 min at room temperature. Subsequently, after cooling to −20°C, 0.72 g (12.4 mmol) rac. methyl oxirane (Merck) was added under stirring. After slowly warming up to room temperature, 20 ml saturated aqueous ammonium chloride was added and the mixture was extracted three times with portions of 50 ml of ether. The combined organic extracts were washed with brine, dried with magnesium sulfate, and concentrated under reduced pressure. The crude product was distilled in vacuo to yield 1.57 g (72%) of **1,** bp_{12}: 90°C.

^{1}H NMR (400.1 MHz, $CDCl_3$): δ = 5.28–5.42 (m, 2H, H-6,7), 3.76–3.84 (m, 1H, H-2), 2.00–2.08 (m, 4H, H-5,8), 1.58 (bs, 1H, OH), 1.40–1.51 (m, 4H, H-3,4), 1.19 (t, 3H, $J_{1,2}$ = 6.4 Hz, H-1), 0.96 (t, 3H, $J_{9,8}$ = 7.6 Hz, H-9). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 131.99 (+), 128.84 (+), 68.05 (+, C2), 38.47 (−), 26.59 (−), 25.47 (−), 23.09 (+, C1), 20.12 (−, C8), 13.94 (+, C9). MS is shown in Figure 2B below.

(2R,6Z)-Nonen-2-ol. The synthesis was essentially the same as for rac. **1.** Commercially available (Aldrich) (*R*)-methyloxirane (0.72 g, 12.4 mmol) yielded 1.1 g (62%) of (2*R*)-**1**; $[\alpha]^{22}$ = −7.9 (c = 4.1 CH_2Cl_2).

(2S,6Z)-Nonen-2-ol. The synthesis was essentially the same as for rac. **1.** Commercially available (Aldrich) (*S*)-methyloxirane (0.72 g, 12.4 mmol) yielded 1.2 g (68%) of (2*R*)-**1**; $[\alpha]^{22}$ = +7.9 (c = 3.3 CH_2Cl_2).

(Z)-6-Nonen-2-one (2). To a solution of 1.0 g (7.0 mmol) of **1** in 45 ml dichloromethane, 2.67 g (7.1 mmol) pyridinium dichromate and 2 g pulverized molecular sieve (4 Å) were added. After vigorous stirring for 4 hr, the mixture was filtered over 20 g silica (Merck 60, 200–240 mesh). The silica was washed with 150 ml of diethyl ether, and the organic solution was concentrated. The residue was chromatographed on 20 g silica (Merck 60, 200–240 mesh, using pentane–diethylether 20:1 as the eluent), yielding 550 mg (55%) of **2.**

^{1}H NMR (400.1 MHz, $CDCl_3$): δ = 5.40 (dtt, 1H, $J_{6,7}$ = 10.8 Hz, $J_{6,5}$ = 7.0 Hz, $J_{6,8}$ = 1.5 Hz, H-6), 5.28 (dtt, 1H, $J_{7,8}$ = 7.0 Hz, $J_{7,5}$ = 1.5 Hz, H-7), 2.43 (t, 2H, $J_{3,4}$ = 7.4 Hz, H-3), 2.13 (s, 3H, H-1), 1.98–2.08 (m, 4H, H-5,8), 1.64 (qu, 2H, $J_{4,5}$ = $J_{4,3}$ = 7.4 Hz, H-4), 0.96 (t, 3H, $J_{9,8}$ = 7.6 Hz, H-9). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 209.04 (o, C2), 132.67 (+), 128.04 (+), 43.05 (−, C3), 29.90 (+, C1), 26.39 (−), 23.39 (−), 23.74 (−), 20.53 (−), 14.32 (+, C9). The mass spectrum is shown in Figure 2A below.

(E)-1-Bromo-3-hexene (8). Starting from 10.0 g (0.10 mol) of commercially available (*E*)-3-hexen-1-ol (**7**), the synthesis was essentially the same as for **6,** yielding 8.8 g (54%) of **8;** bp_{12}: 62–63°C.

^{1}H NMR (400.1 MHz, $CDCl_3$): δ = 5.54 (dtt, 1H, J = 14.8 Hz, J = 6.2 Hz, J = 1.3 Hz), 5.39 (dtt, 1H, J = 14.8 Hz, J = 6.6 Hz, $J_{5,3}$ = 1.5 Hz), 3.37 (t, 2H, $J_{1,2}$ = 7.2 Hz, H-1), 2.54 (m, 2H, H-2), 2.03 (m, 2H, H-5), 0.98 (t, 3H, $J_{6,5}$ = 7.6 Hz, H-6). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 135.84 (+), 125.45 (+), 36.06 (−), 32.90 (−), 25.56 (−), 13.64 (+). MS; *m/z*, 39 (47), 41 (100), 53 (15), 55 (95), 63 (16), 67 (31), 69 (16), 82 (16), 83 (62), 162 (13, M^+), 164 (13, M^+).

rac. (E)-6-Nonen-2-ol (3). In a reaction carried out analogously to the synthesis of rac. **1,** 2.5 g (15.3 mmol) of **8** and 0.72 g (12.4 mmol) methyloxirane yielded 1.4 g (67%) of **3;** bp_{12}: 90°C.

^{1}H NMR (400.1 MHz, $CDCl_3$): δ = 5.35–5.50 (m, 2H, H-6,7), 3.75–3.83 (m, 1H, H-2), 1.96–2.03 (m, 4H, H-5,8), 1.35–1.50 (m, 5H, H-3, 4, OH), 1.09 (t, 3H, $J_{1,2}$ = 6.2 Hz, H-1), 0.96 (t, 3H, $J_{9,8}$ = 7.4 Hz, H-9). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 132.49 (+), 128.88 (+), 68.08 (+, C2), 38.83 (−), 32.48 (−), 25.76 (−), 25.59 (−) 23.50 (+, C1), 13.97 (+, C9). MS; *m/z*, 39 (30), 41 (65), 43 (35), 45 (55), 53 (10), 55 (28), 58 (14), 67 (100), 68 (18), 71 (36), 81 (13), 82 (59), 95 (15), 109 (4), 113 (1), 124 (1), 127 (1), 142 (4, M^+).

(E)-6-Nonen-2-one (4). The synthesis was essentially the same as for **2,** 350 mg (2.5 mmol) of **3** yielded 250 mg (72%) of **4.**

^{1}H NMR (400.1 MHz, $CDCl_3$): δ = 5.46 (dtt, 1H, J = 15.2 Hz, J = 6.4 Hz, J = 1.4 Hz), 5.34 (dtt, 1H, J = 15.2 Hz, J = 6.6 Hz, J = 1.4 Hz), 2.41 (t, 2H, $J_{3,4}$ = 7.4 Hz, H-3), 2.13 (s, 3H, H-1), 1.96–2.03 (m, 4H, H-5,8), 1.64 (qu, 2H, $J_{4,3}$ = $J_{4,5}$ = 7.4 Hz, H-4), 0.96 (t, $J_{9,8}$ = 7.6 Hz, 3H, H-9). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 209.11 (o, C2), 133.08 (+), 128.16 (+), 43.00 (−, C3), 31.89 (−), 29.92 (+, C1), 25.58 (−), 23.63 (−), 13.91 (+, C9). MS; *m/z*, 39 (23), 41 (39), 43 (91), 54 (10), 55 (18), 58 (13), 67 (100), 71 (9), 82 (42), 97 (3), 111 (6), 125 (3), 140 (3, M^+).

Field Tests. Field trapping was conducted in young birch forests in the surroundings of Lund (Sweden), Turku (Finland), and Kirovsk (Russia) in 1993 and 1994. Synthetic blends were prepared in hexane. Red rubber septa (Arthur H. Thomas Co. Catalog No. 1780-J07) were used as dispensers, and the traps used were Lund II sticky traps (Anderbrant et al., 1989). All traps were hung on the branches of young birch trees 1–2 m above the ground and checked every day. Within a replicate (N = 5–10), traps were spaced at least 5 m apart, and trap positions within a series were randomized at each sampling time to minimize the effects of habitat heterogeneities. Subtractive assays were first carried out in Kirovsk, June 11–15, 1993, to determine the attractivity to males of blends

containing compounds identified from the female pheromone gland extracts. In 1994 different ratios of (2*S*,6*Z*)- and (2*R*,6*Z*)-nonen-2-ol were tested in all three places. Finally, dose–response experiments with (2*S*,6*Z*)-nonen-2-ol were performed in Lund and Turku, whereas different doses of a 3 : 1 mixture of (2*S*,6*Z*)- and (2*R*,6*Z*)-nonen-2-ol were tested in Turku only.

RESULTS

GC-EAD of Female Extracts and Chemical Identification

Eriocrania sangii. GC-EAD analysis of the extracts from female fifth abdominal segment on a DB-WAX column indicated that three compounds elicited strong responses from a conspecific male antenna (Figure 1). The mass spectra of these three GC-EAD active compounds suggested two methylketones as indicated by the fragment *m/z* 58 and a methylcarbinol showing the characteristic *m/z* 45. Comparison of mass spectral data (McLafferty and Stauffer, 1989) and gas chromatographic retention time proved the minor ketone to be nonan-2-one (C_9-2-one). The mass spectrum of the major ketone shown in Figure 2A was known to us from earlier work (Francke et al., 1979; Vanderwel et

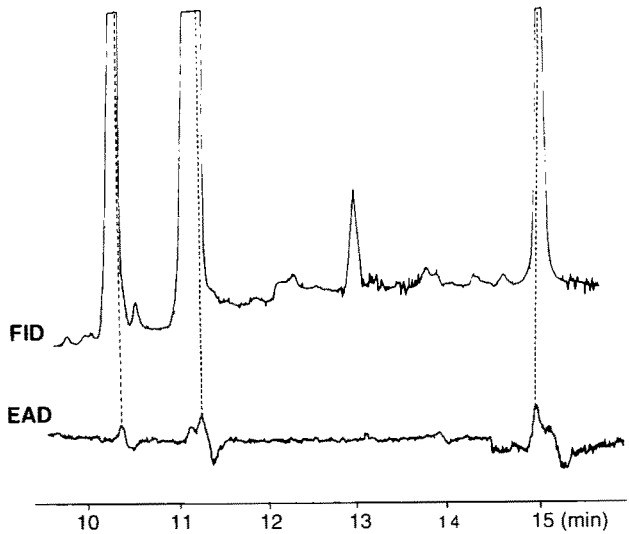


FIG. 1. Simultaneously recorded flame ionization detector (FID) and electroantennographic detector (EAD) responses on a DB-WAX column using antennae of male *Eriocrania sangii* in response to pheromone gland extracts from conspecific females.

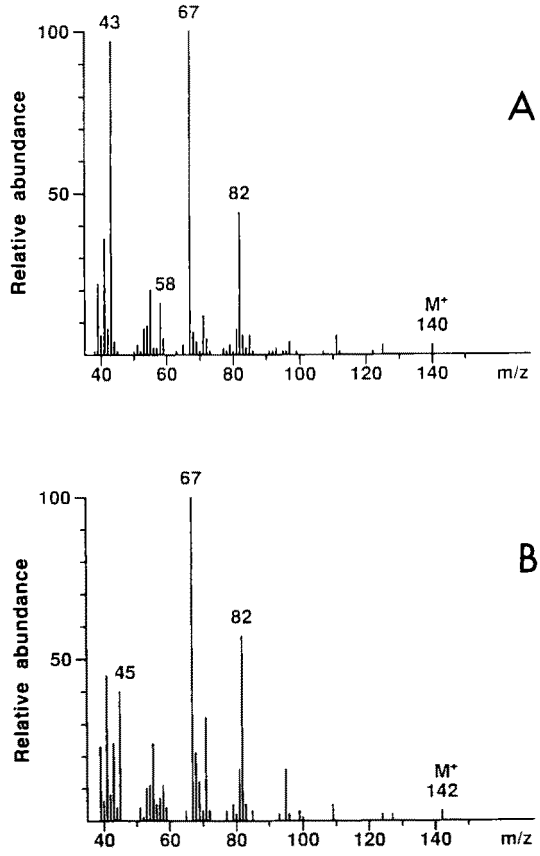


FIG. 2. Mass spectrum of (Z)-6-nonen-2-one (A) and (Z)-6-nonen-2-ol (B) found in female gland extracts of *Eriocrania sangii*.

al., 1992). The compound was clearly shown to be 6-nonen-2-one by the molecular ion at *m/z* 140 and a McLafferty rearrangement producing *m/z* 58 and an intense signal at *m/z* 82, which upon loss of a methyl group gave rise to the formation of the base peak of the spectrum at *m/z* 67. Upon gas chromatography, the double bond was shown to have *Z* geometry since the synthetic (Z)-6-nonen-2-one (Z6-C_9-2-one), which produced the same mass spectrum, coeluted with the natural compound, while the *E* isomer eluted earlier (Figure 3A). The third EAD active compound produced nonan-2-ol upon microhydrogenation (Huwyler, 1973). With intense signals at *m/z* 82 and *m/z* 67, its mass spectrum (Figure

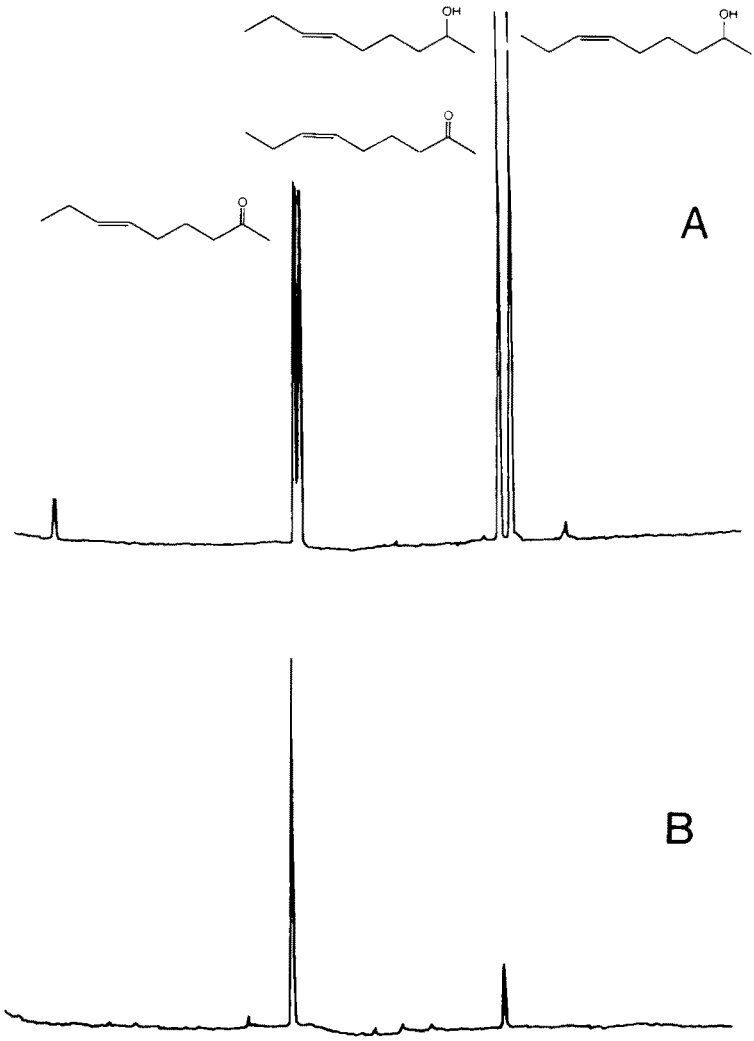


FIG. 3. Gas chromatographic separation of (A) 6-nonen-2-ones and 6-nonen-2-ols, (B) natural products, (C) sample A + sample B on a fused silica capillary column.

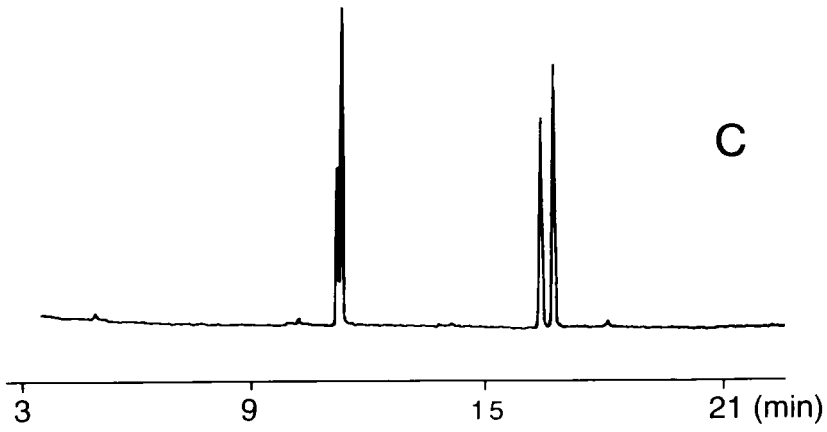


FIG. 3. Continued.

2B) was strikingly similar to that of 6-nonen-2-one, but its molecular mass of *m/z* 142 suggested a nonen-2-ol. Assuming close structural and biogenetic relations between this compound and (*Z*)-6-nonen-2-one, it was tentatively assigned to be (*Z*)-6-nonen-2-ol (Z6-C_9-2-ol). A synthetic sample produced the same mass spectrum as the natural compound and coeluted upon gas chromatography (Figure 3B), while the *E* isomer appeared earlier (Figure 3C). As shown with the synthetic samples, the enantiomers of Z6-C_9-2-ol could be gas chromatographically well separated using a modified cyclodextrin as the stationary phase (Figure 4A). The earlier eluting *S* enantiomer coeluted with the natural product (Figure 4C). We could not exclude the presence of a very small amount of the *R* enantiomer. However, as shown by GC-MS, the minor peak eluting shortly after (*S*)-Z6-C_9-2-ol (Figure 4C) does not represent the *R* enantiomer. Further GC-EAD analyses of female extracts on a cyclodextrin column and using a conspecific male antennae as the detector showed that the *S* enantiomer was found predominantly in the female extract. However, a tiny amount of *R* enantiomer was detected in the extract based on evidence that a compound with the same retention time as the synthetic *R* enantiomer elicited a small, but significant antennal response (Figure 5). The EAD active compounds in the female extract could thus be identified as C_9-2-one, Z6-C_9-2-one, and (*S*)-Z6-C_9-2-ol.

E. semipurpurella. Three compounds with the same retention indices as those found in *E. sangii* elicited strong responses from male antennae in GC-EAD analyses of female extracts on a DB-WAX column (Figure 6). Mass spectra of these three compounds were also the same as those described in *E.*

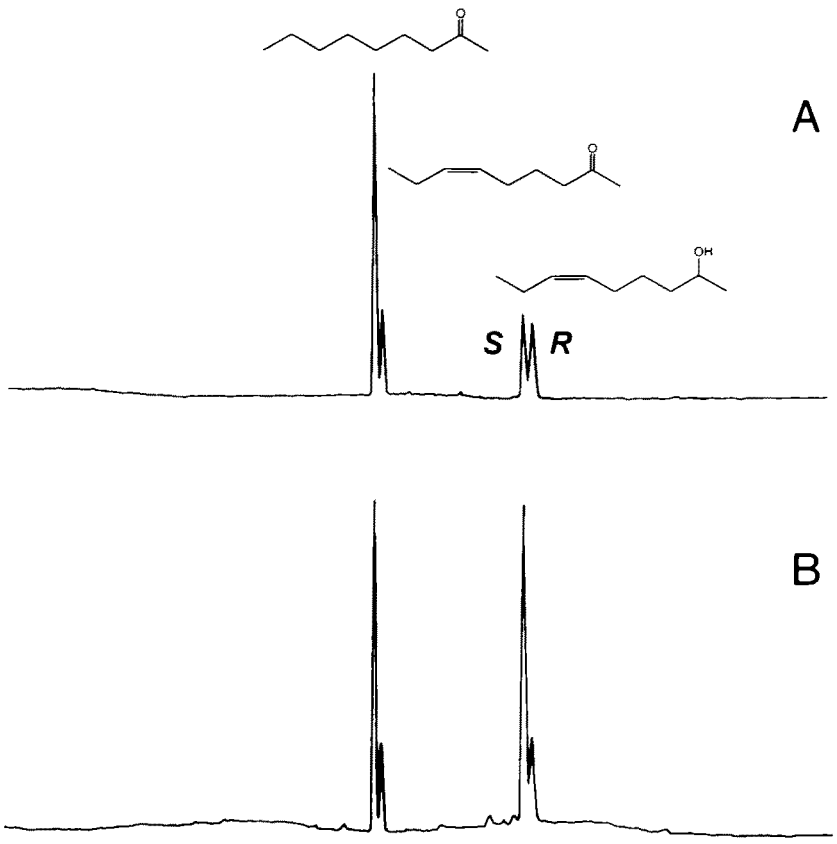


FIG. 4. Gas chromatographic separation of (A) 2-nonanone, (Z)-6-nonen-2-one, and (Z)-6-nonen-2-ols; (B) sample A + (2*S*,6*Z*)-nonen-2-ol; (C) female extracts of *Eriocrania sangii;* (D) sample A + sample C on a cyclodextrin column.

sangii. However, gas chromatography on a chiral cyclodextrin column showed that both *S* and *R* enantiomers of the alcohol, in a relative ratio of 2:1, were found from the analyses of individual female extract (Figure 7). GC-EAD analysis of a synthetic mixture containing the two enantiomers in a relative ratio of 1:0.9 showed that male antennae responded to both enantiomers (Figure 8). The EAD active compounds in the extracts could thus be identified to be C_9-2-one, Z6-C_9-2-one, (*S*)-Z6-C_9-2-ol, and (*R*)-Z6-C_9-2-ol.

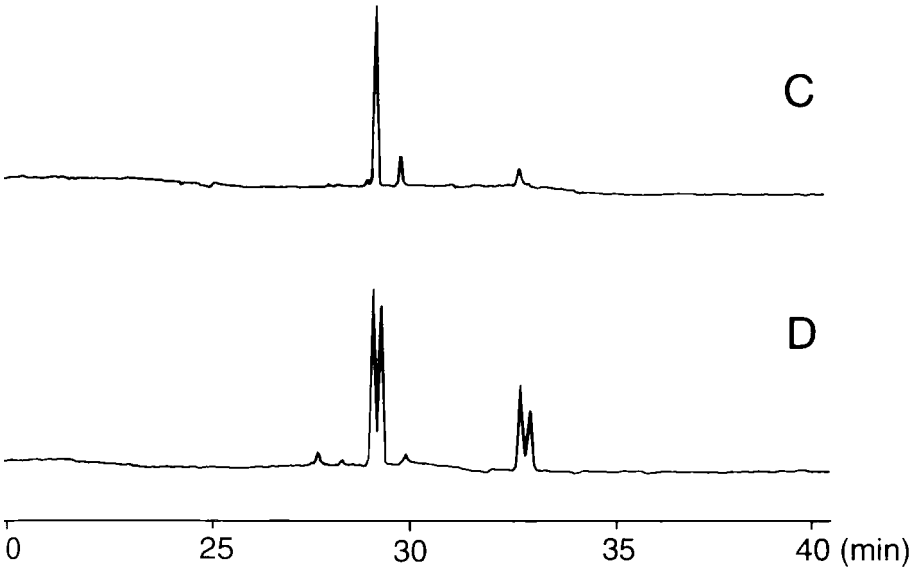


FIG. 4. Continued.

Field Tests. The three synthetic compounds in two different ratios were not attractive to males of either species (Table 1). However, subtraction of the major compound, Z6-C_9-2-one, increased the bait attraction to males of *E. sangii* significantly. The highest number of trapped males was observed when the bait contained pure (*S*)-Z6-C_9-2-ol only. Thus, we concluded that (*S*)-Z6-C_9-2-ol was the only behaviorally attractive sex pheromone component for *E. sangii* males. Males of *E. semipurpurella* were also attracted to the baits containing (*S*)-Z6-C_9-2-ol, but the catch number was extremely low compared to numbers of male *E. sangii* caught in the traps.

The importance of the enantiomeric composition of the alcohol was elucidated in the field tests with different ratios of (*S*)- and (*R*)-Z6-C_9-2-ol (Figure 9). The traps with baits containing pure *S* enantiomer of Z6-C_9-2-ol and its mixture with 5% of the *R* enantiomer were attractive to males of *E. sangii* in all three regions. The bait completely lost its attractivity to males of *E. sangii* by adding more than 20% of the *R* enantiomer in the blend. However, presence of the *R* enantiomer seemed obligatory to attract males of *E. semipurpurella*, and the attractive ratios of chiral alcohols differed in three locations. In southern Sweden a racemic mixture of the two alcohols was the only bait attracting

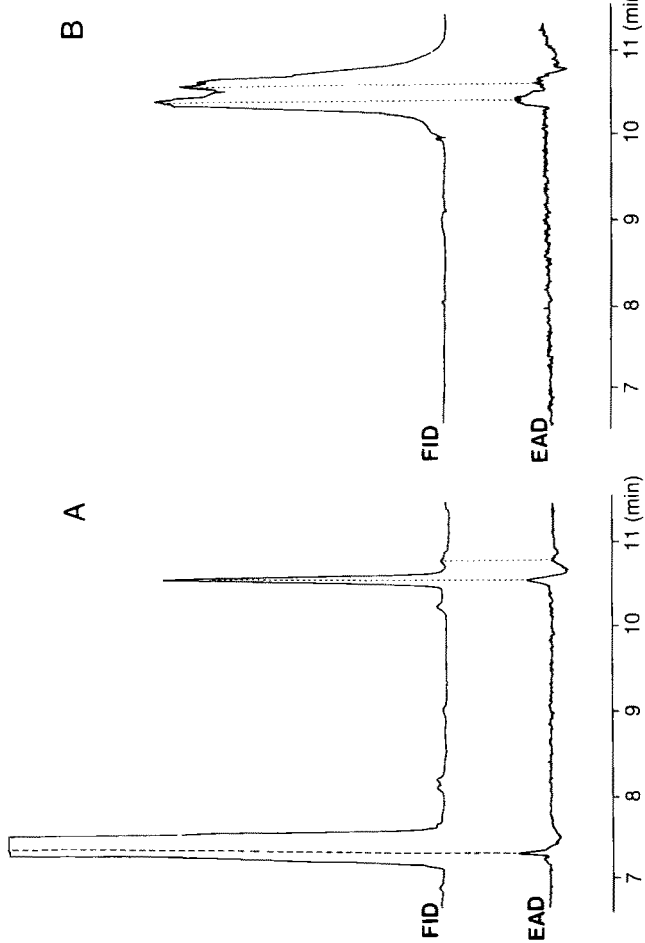


FIG. 5. Simultaneous flame ionization detector (FID) and electroantennographic detector (EAD) responses after separation of a female *Eriocrania sangii* extract (A) and a synthetic mixture of (2*R*,6*Z*)- and (2*S*,6*Z*)-nonen-2-ol (B) on a cyclodextrin column, using an antenna of male *E. sangii* as the detector.

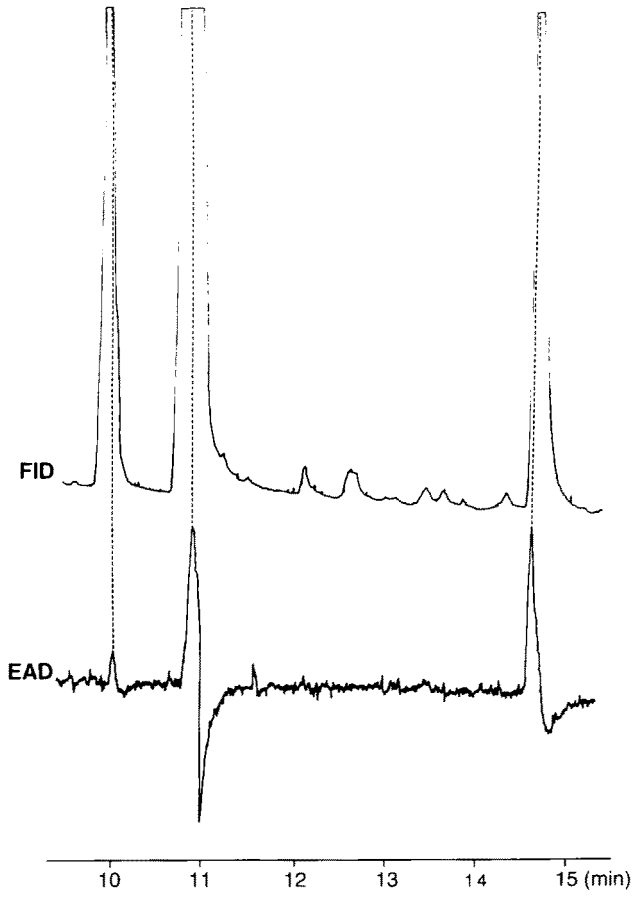


FIG. 6. Simultaneously recorded flame ionization detector (FID) and electroantennographic detector (EAD) responses after separation of a *Eriocrania semipurpurella* extract on a DB-WAX column, using a conspecific antenna as the detector.

significant numbers of *E. semipurpurella*. In the northwest of Russia baits with pure *R* enantiomer or its mixture with 5% of *S* enantiomer caught the largest number of *E. semipurpurella* males. The intermediate situation was discovered in southern Finland, where all examined ratios of (*S*)- and (*R*)-Z6-C_9-2-ol caught a number of male *E. semipurpurella*, but lures containing 50–80% of (*R*)-Z6-C_9-2-ol were more attractive than other tested ratios.

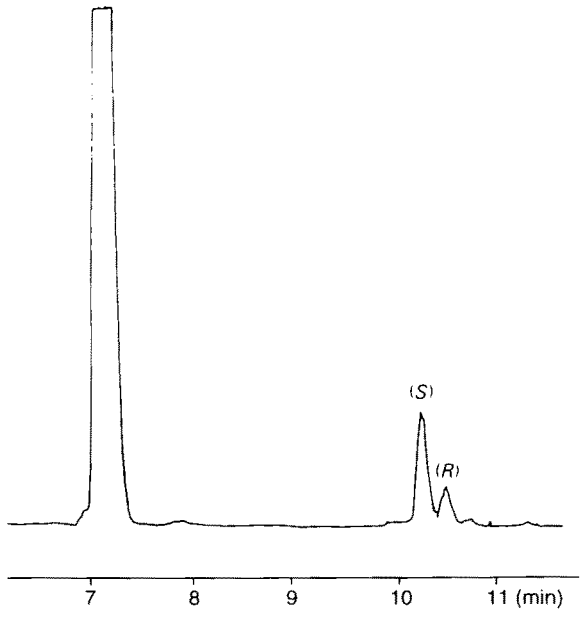


FIG. 7. Chiral gas chromatographic analysis of a female sex pheromone gland extract of *Eriocrania semipurpurella* on a cyclodextrin column operated under the conditions described in the text.

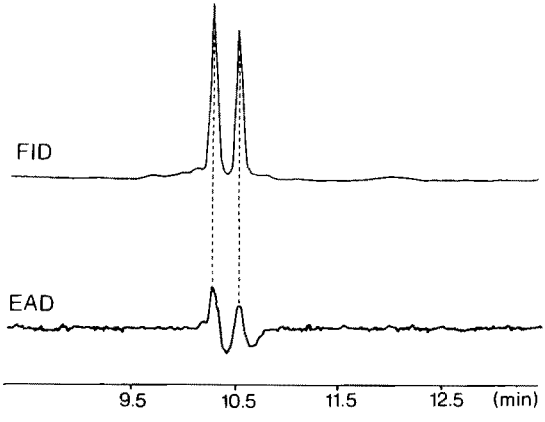


FIG. 8. Simultaneously recorded flame ionization detector (FID) and electroantennographic detector (EAD) responses after separation of a synthetic mixture of (2*R*,6*Z*)- and (2*S*,6*Z*)-nonen-2-ol on a cyclodextrin column, using antennae of male *Eriocrania semipurpurella* as the detector.

TABLE 1. FIELD TRAPPING OF MALE *Eriocrania semipurpurella* AND *E. sangii* IN SUBTRACTIVE ASSAYS OF BLENDS OF THREE PHEROMONE CANDIDATES IN KIROVSK, MURMANSK REGION, JUNE 11–15, 1993

C_9-2-one (μg)	Z6-C_9-2-one (μg)	(*S*)-Z6-C_9-2-ol (μg)	Catch per trap (mean ± SE)[a]	
			E. semipurpurella	*E. sangii*
A[b]				
6.8	100	2.7	0.2 ± 0.2b	0 ± 0b
6.8	100		0 ± 0b	0 ± 0b
	100	2.7	0 ± 0b	0.2 ± 0.2b
6.8		2.7	0.2 ± 0.2b	5.2 ± 2.4a
	100		0 ± 0b	0.2 ± 0.2b
		2.7	1.2 ± 0.4a	9.4 ± 1.9a
6.8			0 ± 0b	0 ± 0b
B[c]				
3.3	100	6.7	0 ± 0a	0 ± 0b
3.3	100		0 ± 0a	0.2 ± 0.2b
	100	6.7	0 ± 0a	0 ± 0b
3.3		6.7	0.8 ± 0.6a	26.6 ± 3.6a
	100		0 ± 0a	0 ± 0b
		6.7	0.8 ± 0.4a	32.8 ± 7.2a
3.3			0.4 ± 0.4a	0 ± 0b

[a] Means followed by the same letter within series A and B, respectively, are not significantly different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello robust Mann-Whitney U test ($P < 0.05$), $N = 5$.
[b] Ratios of the three pheromone candidates are the same as those found in the female extracts of *E. semipurpurella*.
[c] Ratios of the three pheromone candidates are the same as those found in the female extracts of *E. sangii*.

A mixture of the (*S*)- and (*R*)-Z6-C_9-2-ol in a ratio of 3 : 1 and the pure *S* enantiomer were also tested in different doses (Table 2). The higher doses (10–1000 μg) of the pure *S* enantiomer caught the largest number of males *E. sangii*. Males of *E. semipurpurella* were trapped in Finland only, and the 1000-μg dose of the enantiomeric mixture was the most attractive.

DISCUSSION

The present investigation of *Eriocrania semipurpurella* and *E. sangii* confirms that the chemical structures of sex pheromones in a primitive lineage of Lepidoptera are different from those in the ditrysian moths. The two species

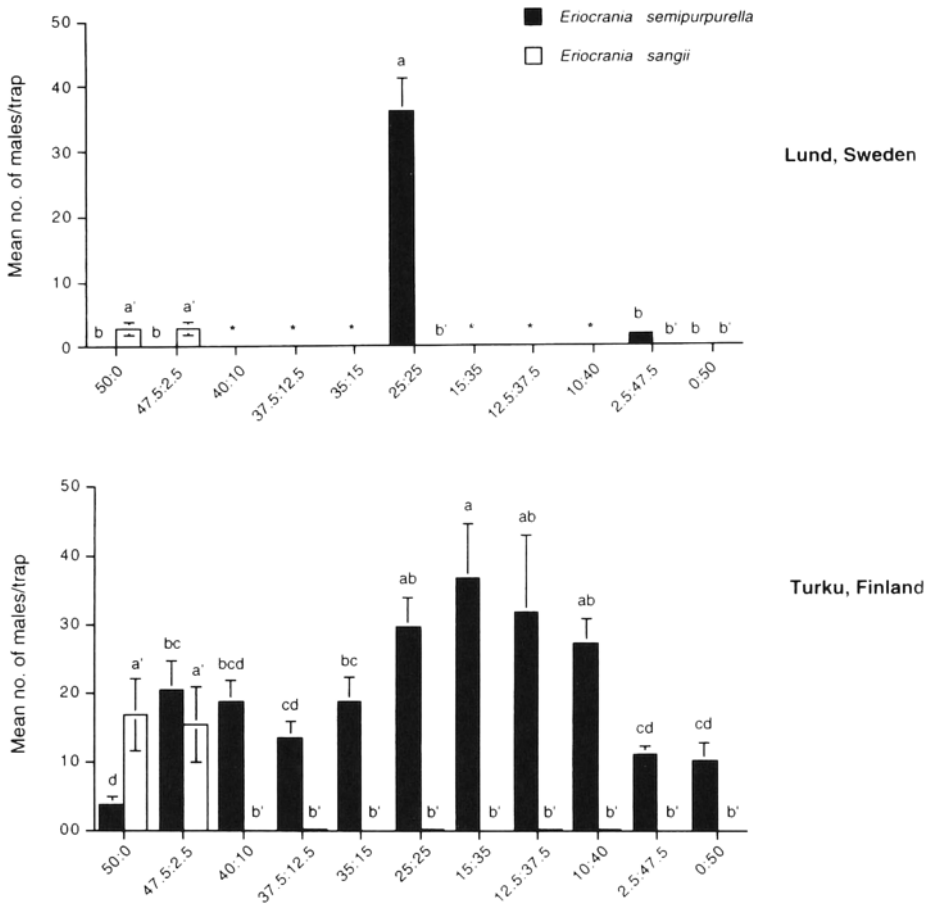


FIG. 9. Geographical variation in mean number of male moths (±SE) trapped with baits containing different enantiomeric ratios of (2*R*,6*Z*)- and (2*S*,6*Z*)-nonen-2-ol, as observed in field tests conducted at three locations in 1994. Bars accompanied by the same letters represent means that are not statistically different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello Mann-Whitney U test ($P < 0.05$), $N = 5$. Blends indicated with an asterisk were not tested.

investigated, as well as *E. cicatricella* studied earlier (Zhu et al., 1995), produce short-chain ketones and alcohols as pheromone components that are similar to sex pheromone components earlier found in Trichoptera (Löfstedt et al., 1994).

The alcohols found in *E. semipurpurella* and *E. sangii* are likely to be the reduction products of corresponding methylketones. The antagonistic behavioral

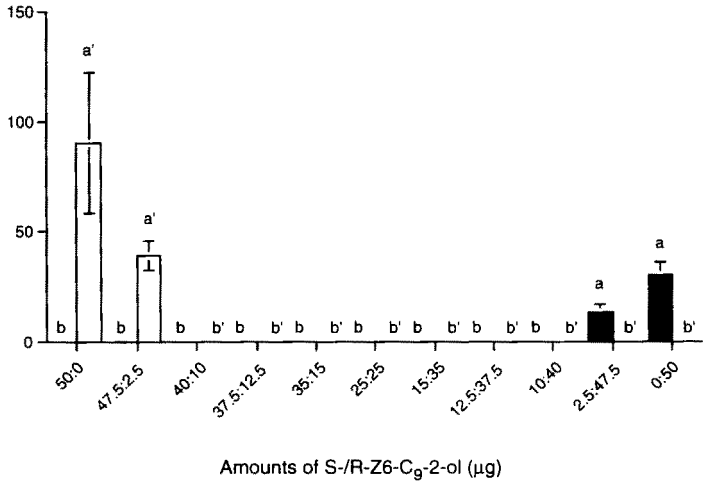


FIG. 9. Continued.

activity of Z6-C_9-2-one when tested in mixtures with the alcohols strongly suggests that the ketones are precursors of the alcohols and that they are not released by calling females under natural conditions. Methyl ketones are widespread gland constituents among Hymenoptera (Blum, 1981), and Z6-C_9-2-one together with *exo*-brevicomin (Silverstein et al., 1968) has been identified as a male-specific component in the ash bark beetle, *Leperisinus varius* (Francke et al., 1979). Francke (1981) postulated a biogenetic relationship between these two compounds and regarded the unsaturated ketone as a precursor of brevicomin, since enzymatic epoxidation of the ketone followed by ring closure would directly yield brevicomin (see also Kohnle et al., 1992). This route was recently confirmed by Vanderwel et al. (1992) in an *in vivo* experiment in the mountain pine beetle, *Dendroctonus ponderosae*, based on evidence that the beetles transform labeled Z6-C_9-2-one into *exo*-brevicomin. Interestingly, secondary alcohols such as 2-tridecanol and 2-pentadecanol with the *S* configuration were identified from the defensive secretion of larvae of two notodontid caterpillars (Attygalle et al., 1993). In addition, (2*S*)-alkan-2-ols were found to be components of the sex pheromone of queens of the stingless bee, *Scaptotrigona postica* (Engels et al., 1990).

The fact that closely related species of insects commonly use similar pheromone components may seem to limit their use in species-specific communication. However, the signal may be specific not only because of compounds involved in a given blend, but also due to the ratio between pheromone components, including the enantiomeric composition of optically active components.

TABLE 2. DOSE-RESPONSE TEST OF SYNTHETIC PHEROMONE CANDIDATES FOR *Eriocrania sangii* AND *E. semipurpurella* IN LUND, SWEDEN, APRIL 26–29, 1994 AND TURKU, FINLAND, MAY 5–9, 1994

(2*S*,6*Z*)-nonen-2-ol (μg)	(2*R*,6*Z*)-nonen-2-ol (μg)	Catch per trap (mean ± SE)[a]	
		E. sangii	*E. semipurpurella*
Lund, Sweden, April 26–29, 1994			
0.1		0.2 ± 0.2b	0 ± 0c
1		1.6 ± 1.4ab	0 ± 0c
10		1.4 ± 1.2ab	0 ± 0c
100		7.6 ± 4.0a	0 ± 0c
1000		5.4 ± 3.0ab	0 ± 0c
Turku, Finland, May 5–9, 1994			
0.1		0 ± 0c	0.6 ± 0.3ab
1		1.4 ± 0.5c	0 ± 0b
10		8.6 ± 1.9a	2.2 ± 0.9ab
100		17.6 ± 4.8a	2.4 ± 1.3ab
1000		16.0 ± 2.3a	3.0 ± 0.8a
Turku, Finland, May 5–9, 1994			
0.075	0.025	0 ± 0a	0.4 ± 0.3c
0.75	0.25	0 ± 0a	1.4 ± 0.5c
7.5	2.5	0 ± 0a	2.6 ± 0.9c
75	25	0 ± 0a	12.8 ± 0.6b
750	250	0.2 ± 0.2a	39.0 ± 6.3a

[a]Means within each column in each experiment followed by the same letter are not significantly different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello robust Mann-Whitney U test ($P < 0.05$), $N = 5$.

Chirality plays a very important role in the specificity of many pheromones, and often one enantiomer gives a positive biological response while the other enantiomer acts as an inhibitor. In the present study males of *E. sangii* were attracted by the baits containing pure *S* enantiomer or a mixture with less than 5% of *R* enantiomer. The attractivity was decreased significantly after addition of more than 5% of the *R* enantiomer. Females of *E. sangii* produce mainly the *S* enantiomer as their pheromone, while female extracts of *E. semipurpurella* contain both *R* and *S* enantiomers. The pheromone of *E. cicatricella*, another species in the same genus, was identified as (2*R*,4*Z*)-hepten-2-ol. No males of *E. cicatricella*, but a large number of *E. sparrmannella*, were caught in traps baited with (2*S*,4*Z*)-hepten-2-ol (Zhu et al., 1995).

Enantiospecific activity has been well documented in several other species of Lepidoptera. (3*Z*,9*Z*-6*S*,7*R*)-epoxy-nonadecadiene is used as a sex pheromone

component by *Erannis defoliaria*. However, the addition of its 6*R*,7*S* antipode significantly decreases male catches in the field. In contrast, this compound is very attractive to males of another geometrid moth, *Colotois pennaria* (Szöcs et al., 1993). The sex pheromone of the gypsy moth, *Lymantria dispar*, was identified as (*Z*)-7,8-epoxy-2-methyloctadecane (Bierl et al., 1970). Studies using (7*R*,8*S*)-(+) and (7*S*,8*R*)-(−) enantiomers and the racemic mixture indicated that males only respond to the (+) enantiomer and are inhibited by very small amounts of (−)-disparlure (Cardé et al., 1977). Hansen (1984) reported that females of the nun moth, *L. monacha*, produced a 90:10 blend of (−)- and (+)-disparlure, and male moths responded to both of the enantiomers but were mostly attracted to the mixture (Klimetzek et al., 1976; Vité et al., 1976).

E. semipurpurella and *E. sangii* are difficult to distinguish morphologically, even when the male genitalia are investigated (Heath, 1976). Recently, however, the form of the terminal segment of the maxillary palp was demonstrated as a reliable external character to distinguish between *E. semipurpurella* (pointed apex) and *E. sangii* (forked apex). The difference in female pheromone composition and in male pheromone response among the two forms correlated to this morphological difference, which supports the notation that *E. semipurpurella* and *E. sangii* are two different species, although closely related to each other.

Geographical differences in responses to pheromones have earlier been reported in both Lepidoptera and Coleoptera. Behaviorally significant variation in pheromone composition has been shown among populations of the turnip moth, *Agrotis segetum* (Arn et al., 1983; Löfstedt et al., 1986; Tóth et al., 1992). The largest difference in attraction of males appeared between populations north and south of the Sahara Desert. Pure (*Z*)-5-decenyl acetate is not attractive to males in most of the European populations investigated but is very attractive to males in two South African populations. This male behavioral difference corresponds to significant differences in female pheromone production between European and African populations (Wu, Cottrell, Hansson, and Löfstedt, unpublished results). Both inter- and intrapopulational variation of ipsdienol configuration have been noted in the pine engraver, *Ips pini*. Populations of *I. pini* in Idaho (Plummer et al., 1976) and California produce primarily (−)-ipsdienol and also preferentially respond to this component, while the New York population specifically produces and responds to a 65:35 mixture of (+)-:(−)-ipsdienol (Lanier et al., 1980). The different pheromone response profiles among males of *E. semipurpurella* at three locations in northern Europe is another example of geographic pheromone variation with respect to enantiomeric composition of the pheromone. Males from Lund, in the south of Sweden and from Turku, in the south of Finland, respond to different mixtures of (*R*)- and (*S*)-*Z*6-C_9-2-ol, whereas males from the population in Kirovsk, northwest Russia, are specifically attracted to the pure *R* enantiomer. The *R* enantiomer does not

attract any males in Lund, whereas in Turku some males were found in the traps baited with this compound, albeit in significantly lower numbers compared to traps baited with the mixtures. Female extracts of *E. semipurpurella* collected in Lund were found to contain mixtures of *R* and *S* enantiomers in a ratio of 1:2. We were not able to collect females from Kirovsk for pheromone analysis, but based on the field-trapping tests we expect females from this area to produce mainly the *R* enantiomer.

Foster et al. (1987) analyzed pheromone production and response among some geographical populations of New Zealand tortricids and discovered several sibling species within the genera *Ctenopseustis* and *Planotortrix*. The differences in response profiles among geographical populations of *E. semipurpurella* and the apparent bimodal distribution of trap catches among baits with different enantiomeric composition in Finland suggest that we are actually dealing with a complex of sibling species. This possibility will be investigated by pheromone analysis and morphological investigations of *E. semipurpurella* from more places in Sweden, Finland, and Russia.

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LOCATION AND BEHAVIORAL EFFECTS OF LECTIN BINDING ON ENTOMOPATHOGENIC NEMATODES WITH DIFFERENT FORAGING STRATEGIES

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Abstract—Lectins have been used to locate and ascribe a function to sensory organs of nematodes. We had two objectives: (1) to determine the location of carbohydrate exudates on the surface of three species of entomopathogenic nematodes with different foraging strategies and (2) to evaluate the importance of carbohydrates in host finding and recognition. *Steinernema carpocapsae* finds hosts by ambushing, and most individuals respond poorly to host cues. No specific areas of lectin binding were recorded for this species. Wheat germ agglutinin bound specifically to the anterior region of *Heterorhabditis bacteriophora* and to the anterior and posterior of *S. glaseri*. These two nematode species are cruise foragers and are highly responsive to host cues. The behavioral effects of lectins varied. None of the species' responses to volatile host cues was affected. *S. carpocapsae*'s rate of attachment during ambushing was increased by treatment with WGA, and this effect was negated by treatment with a competing sugar (*N*-acetylglucosamine). The infectivity of *S. glaseri* in an assay that required movement through 5 cm of sand to find the host was significantly decreased by treatment with WGA.

Key Words—Entomopathogenic nematodes, behavior, foraging, lectins, insects, biological control, ecology.

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INTRODUCTION

Most parasitic nematodes find and assess hosts via chemical cues. Information on host quality and location is detected by chemosensilla, primarily the anteriorly located amphids (Ward, 1978), situated in body pores filled with glycoprotein exudates (Aumann, 1993). Behavioral responses to host cues or food resources have been described in detail for plant-parasitic (Pline and Dusenbery, 1987; Grundler et al., 1991), insect-parasitic (Gaugler et al., 1980; Lewis et al., 1992, 1993; Khlibsuwan et al., 1992), and free-living nematodes (Grewal and Wright, 1991; Andrew and Nicholas, 1976). The location and role of nematode sensory organs and their associated glycoprotein exudates have been shown by scanning electron micrography (Forrest et al., 1988; Bird and Bird, 1991) and studied indirectly by labeling with lectins (Zuckerman, 1983; Bone and Bottjer, 1985; Jansson and Nordbring-Hertz, 1984; Jansson, 1993).

Studies using lectins to inhibit behavioral responses of nematodes have elucidated many aspects of their sensory capabilities. Lectins are proteins or glycoproteins that show a specific affinity for a particular sugar residue, and they sometimes inhibit behavioral responses to cues from chemicals that bind to sensilla-produced glycoproteins. Comparative studies of closely related nematode species with different sensory requirements, however, are lacking. Here we use lectins to label the carbohydrate exudates and to determine their role in the infection process for three species of entomopathogenic nematodes with different foraging strategies.

Entomopathogenic nematodes are lethal parasites of insects. The infective stage finds a suitable host and penetrates the hemocoel through natural openings. After penetration, the nematodes release symbiotic bacteria in the genus *Xenorhabdus* (for steinernematids) or *Photorhabdus* (for heterorhabditids). The host is killed within 48 hr by a combination of septicemia, bacteria-produced toxins (Akhurst and Boemare, 1990), and nematode-produced toxins (Burman, 1984). Developing nematodes consume the bacteria and degrading host tissue, produce two to three generations inside the host cadaver, and produce infective juveniles 7–10 days after the initial infection.

Infective stage juvenile entomopathogenic nematodes depend upon chemical cues to find and assess hosts. The importance of different types of cues varies, depending upon the nematode's foraging strategy (Lewis et al., 1995). Cruise foraging nematodes rely on volatile cues for long-range host location and on contact cues for short-range information (Lewis et al., 1992, 1993). *Heterorhabditis bacteriophora* and *Steinernema glaseri* have many characteristics of cruise foragers. At the other end of the foraging continuum is *Steinernema carpocapsae*, which primarily ambushes hosts by nictation, a behavior in which nematodes lift the anterior 95% of their body from the substrate to facilitate attachment to passing hosts (Campbell and Gaugler, 1993). Attachment to hosts

does not appear to depend upon any behavioral response to host chemicals on the nematode's part. *Steinernema carpocapsae* is relatively unresponsive to volatile long-range host cues (Lewis et al., 1993). The difference in the importance of chemical cues to host finding among these species suggests that the level of involvement of sensory organs and their exudates varies during host finding.

We had two aims: (1) to determine the location of carbohydrate exudates on the surface of these three species of entomopathogenic nematodes and (2) to measure the degree of involvement (if any) of these carbohydrates in host finding and recognition.

METHODS AND MATERIALS

Nematode Culture. Nematodes were reared in last-instar *Galleria mellonella* after the method of Dutky et al. (1964) at ambient room temperature (21–23°C). Infective-stage juveniles were harvested from white traps (Woodring and Kaya, 1988), after which nematodes were stored at ambient temperature for one to four weeks before testing. Approximately 24 hr before treatment with lectins, all nematodes were desheathed by concentrating them on a membrane filter and allowing them to crawl into distilled water, where they were trapped.

Lectin Treatment. Desheathed infective juveniles of each nematode species were washed three times in phosphate buffer saline (PBS), pH 7.4, and then incubated for 30 min at room temperature with wheat germ agglutinin (WGA) for most behavioral assays and binding location experiments. Soybean agglutinin (SBA) and Osage orange agglutinin (MPA) were additional test lectins in the *S. carpocapsae* attachment assays (see below). Lectins conjugated with fluorescein (FITC) or rhodamine isothiocyanate (TRITC) were obtained from Sigma (St. Louis, Missouri) or EY Labs (San Mateo, California) and used at concentrations of 100 μg lectin/ml in PBS. The treated infective juveniles were washed three times in PBS following the incubation period. Live nematodes with fluorescent-conjugated WGA were then mounted under cover slips on glass microscope slides and examined by incident fluorescence microscopy with excitation and emission filters specific to FITC or TRITC. Control nematodes were incubated with WGA and 0.1 M *N*-acetylglucosamine simultaneously, and examined similarly.

Behavioral Assays. Infective juvenile nematodes were treated as described above for all lectin bioassays. Because of the varied foraging behaviors of these nematode species, four types of bioassays were performed to determine the effects of lectin binding on nematode behavior. Two of the assays measured attraction to host-produced volatiles on an agar substrate and two measured a combination of host finding and establishment of an infection in a host in either a three-dimensional sand matrix or on a two-dimensional surface. Each assay

described below was designed to demonstrate treatment effects on nematodes with particular foraging strategies, so not all assays were performed with all nematode species.

Attraction of nematodes to host volatiles was measured with an agar/plate assay developed by Andrew and Nicholas (1976) to test for attraction to bacteria, later modified by Lewis et al. (1993). Briefly, we tracked infective juveniles' attraction to volatiles in quartered 100-mm Petri dishes filled with 2% agar and marked with concentric rings at 1-cm intervals. At the edge of one quarter were two *G. mellonella* larvae housed in a 1-ml pipet tip suspended approximately 0.5 cm above the agar. The opposite quarter served as the control direction. Nematodes in each section of the two opposite quadrants were counted at 5, 10, and 20 min after inoculation for *S. glaseri* and *S. carpocapsae* or 5, 10, and 30 min for *H. bacteriophora*. An index was calculated to yield the average movement in centimeters towards the host per individual infective juvenile (Lewis et al., 1993).

The quadrant plate assay was used in two ways. First, the attraction of *H. bacteriophora*, *S. glaseri*, and *S. carpocapsae* to *G. mellonella* volatiles was measured with and without lectin treatment. *Steinernema carpocapsae* has been shown to respond poorly to host volatiles (Lewis et al., 1993). However, when cues were offered in natural order, first contact followed by volatiles, *S. carpocapsae* were attracted strongly to host volatiles (Lewis et al., 1995). For the second quadrant plate assay, we collected desheathed *S. carpocapsae* on a membrane filter and wiped about 1000 individuals onto the cuticle of a *G. mellonella* larva with a probe. After 30 min, the nematodes were washed from the insect cuticle with distilled water and then treated as described above for 30 min with WGA or a PBS control. Nematodes were then inoculated into the assay chamber, and their attraction to hosts was assessed. These tests were repeated four times. All data from the quadrant plate bioassay were analyzed with multiple variate analysis for repeated measures (MANOVA; SAS, 1987).

To test the effect of lectin binding on ambushing efficiency, we used a chamber designed to allow nictation and attachment of *S. carpocapsae* to mobile hosts (Campbell and Gaugler, 1993). Petri plates (100 mm) with filter papers moistened with 250 μl deionized water served as the chamber. We sprinkled sand (particle size > 1 μm) on the filter paper to provide a substrate upon which the nematodes could nictate. After treatment with lectins or control solutions, nematodes (either 500 or 1000 individuals) were released into the chamber. Thirty minutes later, one *G. mellonella* was released into the chamber. After a 30-min exposure to nematodes, the insects were removed, the nematodes were washed into separate dishes, and the insect larvae were held for 24 hr and dissected. Nematodes either washed from the insect cuticle or dissected from the hemocoel were counted as successful. Four lectin treatments were tested for

their affect on *S. carpocapsae* attachment to hosts: (1) nematodes exposed to one of three lectins (WGA, SBA, MPA) and a control for 30 min; (2) nematodes exposed to WGA for 15, 30, or 60 min; (3) nematodes exposed to heated (denatured) WGA vs. non-heated WGA; and (4) combinations of hosts and nematodes treated for 30 min with WGA.

We used a sand column assay to determine the effects of lectin (WGA only) binding on the ability of *S. glaseri* to find and infect hosts. The control treatment was nematodes incubated with PBS only. Cylindrical plastic containers (5 cm diam) were filled with 5 cm of moist sand. At the bottom of the column was one *G. mellonella* last instar. Approximately 1000 infective juveniles in water were applied to the top of the column. Sixteen hours later the insects were removed from the assay chamber, washed with water and held two days for dissection. The nematodes washed from the cuticle were counted immediately. After two days of incubation at 25°C, the insects were dissected and all nematodes inside were counted. Data are reported as the percent of the original inoculum that located the insect, including nematodes washed from the cuticle and those that penetrated into the hemocoel.

Unless otherwise specified, all data were analyzed by analysis of variance, and means were separated with the Student-Newman-Keuls test at $\alpha = 0.05$ (SAS, 1987). Percent data were transformed by arcsin (X) before analysis. All means are presented as mean $\pm$ SEM.

RESULTS

Location of Glycoconjugate Exudates. WGA bound specifically to the anterior end of the cruise foraging species *S. glaseri* (Figure 1A) and *H. bacteriophora* (Figure 1B) and generally to the cuticle of the ambush foraging species *S. carpocapsae* (Figure 1C). Most behavioral assays, with the exception of attachment of *S. carpocapsae*, were conducted with WGA only. In all cases, when nematodes were incubated with the lectin plus the competing carbohydrate, no lectin binding occurred.

Behavioral Assays. Lectin binding had various effects on *S. carpocapsae* host-finding behavior, depending on the lectin used and the assay in which the effect was tested. The rate of attachment to mobile hosts in a nictation arena was increased (nearly doubled) by nematode exposure to SBA and WGA when compared to the control, but no such increase was caused by MPA (Figure 2). All subsequent tests were performed with WGA only. WGA affected *S. carpocapsae* attachment differently when the nematodes, the host, or both were treated. Treatment of *G. mellonella* with lectin significantly decreased the rate of attachment by *S. carpocapsae*, whether or not the nematodes were treated

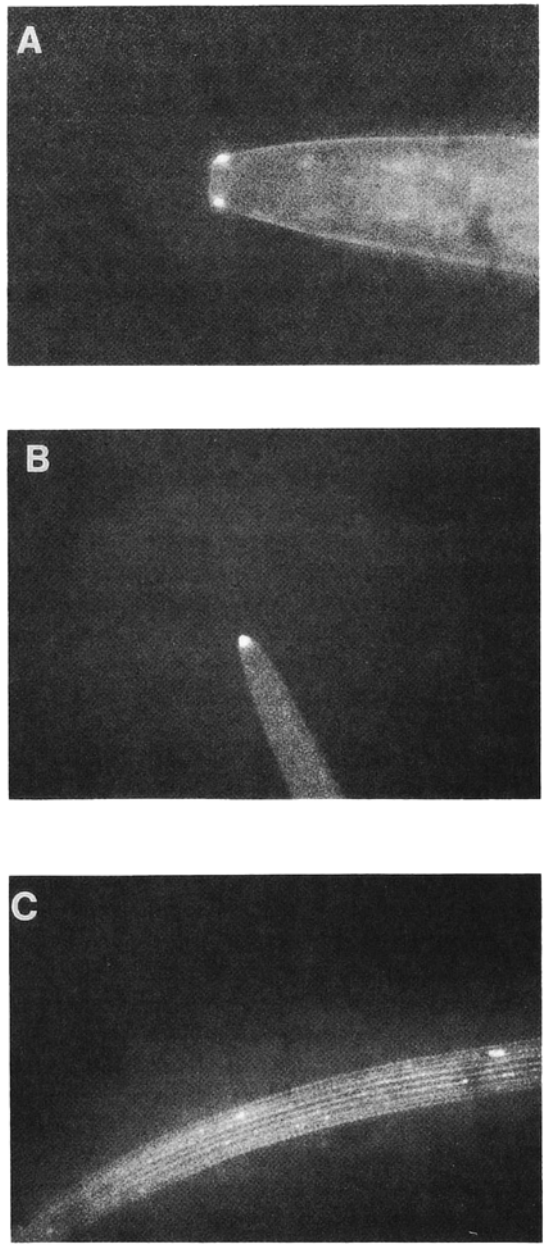

FIG. 1. FITC-labeled wheat germ agglutinin (WGA) binding sites on (A) *S. glaseri*, (B) *H. bacteriophora*, and (C) *S. carpocapsae*.

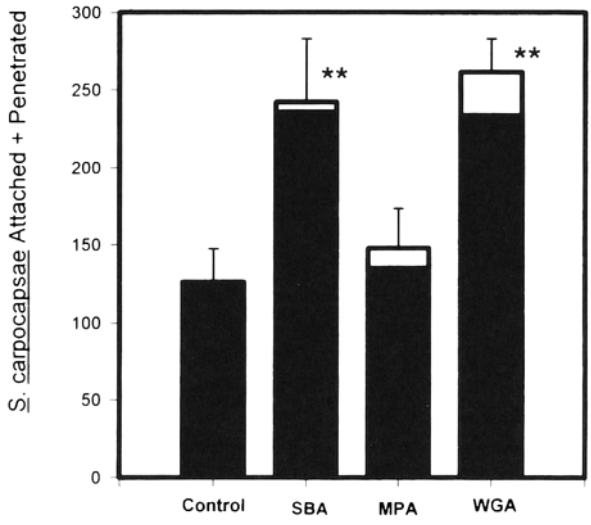

FIG. 2. Effect of lectin treatments of nematodes on *S. carpocapsae* attachment to hosts. SBA = soybean agglutinin, MPA = *Malcura pomifera* agglutinin, and WGA = wheat germ agglutinin. **Treatments significantly different from the control. Shaded areas represent nematodes washed from host cuticle, clear areas represent nematodes dissected from host hemocoel.

with the lectin (Figure 3). The effect of WGA on *S. carpocapsae* attachment to hosts was nullified when the lectin was denatured by heating.

There was no effect of lectin treatment on nematode response to host volatiles for any of the species tested (Figures 4A–C). *Steinernema glaseri* and *H. bacteriophora* continued to be strongly attracted to host volatiles regardless of WGA treatment, whereas *S. carpocapsae* was not attracted before or after lectin exposure. We also tested the attraction of *S. carpocapsae* to volatiles after contact with host cuticle, but again the level of attraction remained unchanged regardless of lectin treatment (Figure 4D).

When challenged with finding and parasitizing a host in a sand column assay, WGA treatment significantly decreased *G. mellonella* parasitization by *S. glaseri*. Control nematodes found hosts at a rate of 24.8 ± 1.65%, whereas only 13 ± 2.6% of WGA-treated nematodes found the host.

DISCUSSION

The location of the binding sites of WGA corresponded with what we predicted on the basis of nematode foraging strategy. Cruise foragers (*S. glaseri* and *H. bacteriophora*) are responsive to host cues, and the areas of their sensory

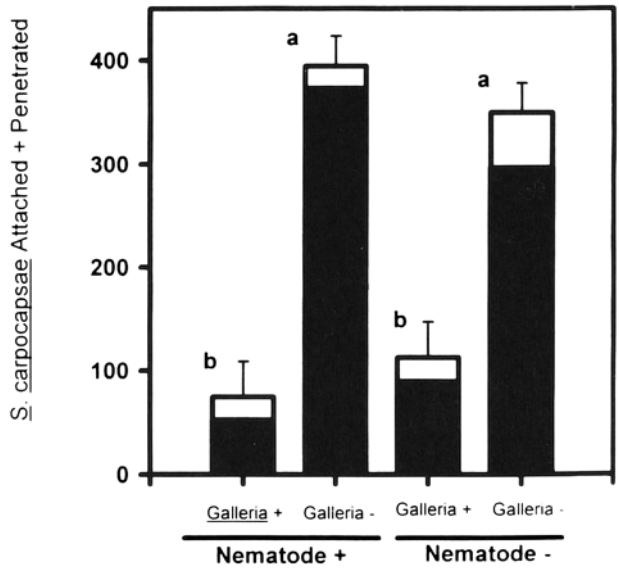

FIG. 3. Effect of wheat-germ agglutinin (WGA) treatment of nematodes, *G. mellonella*, or both on attachment of *S. carpocapsae* to hosts. Galleria +: insects treated with WGA. Galleria −: insects treated with PBS (phosphate buffered saline control). Nematode +: nematodes treated with WGA. Nematode −: nematodes treated with PBS. Bars with the same letter are not significantly different. Shaded areas represent nematodes washed from host cuticle, clear areas represent nematodes dissected from host hemocoel.

organs were labeled with the lectin. Nematode sensory organs produce carbohydrates that are associated with chemotaxis (Zuckerman, 1983). *Steinernema carpocapsae*, an ambush forager, was relatively unresponsive to host cues when they were offered singly (Lewis et al., 1992; 1993), and WGA did not bind to their sensory organs. Species differences in the location of lectin binding sites are not uncommon among nematodes. Bone and Bottjer (1985) compared the location of binding of *Lens culinaris* agglutinin to three gastrointestinal parasite sexual stages and found that all body regions showed binding in *Trichostrongylus colubriformis* and *Haemonchus contortus*, but the head and tail regions of *Nippostrongylus brasiliensis* did not show binding. The differences we have found may be related to the responses to host cues of the nematodes tested.

Effects of lectin binding on the host-finding ability of nematodes have been inconsistent. The first report of lectins mediating nematode chemotaxis was that treatment of *Panagrellus redivivus* with limulin decreased nematodes' response to the nematophagous fungus *Drechmeria coniospora* by binding to sialic acid

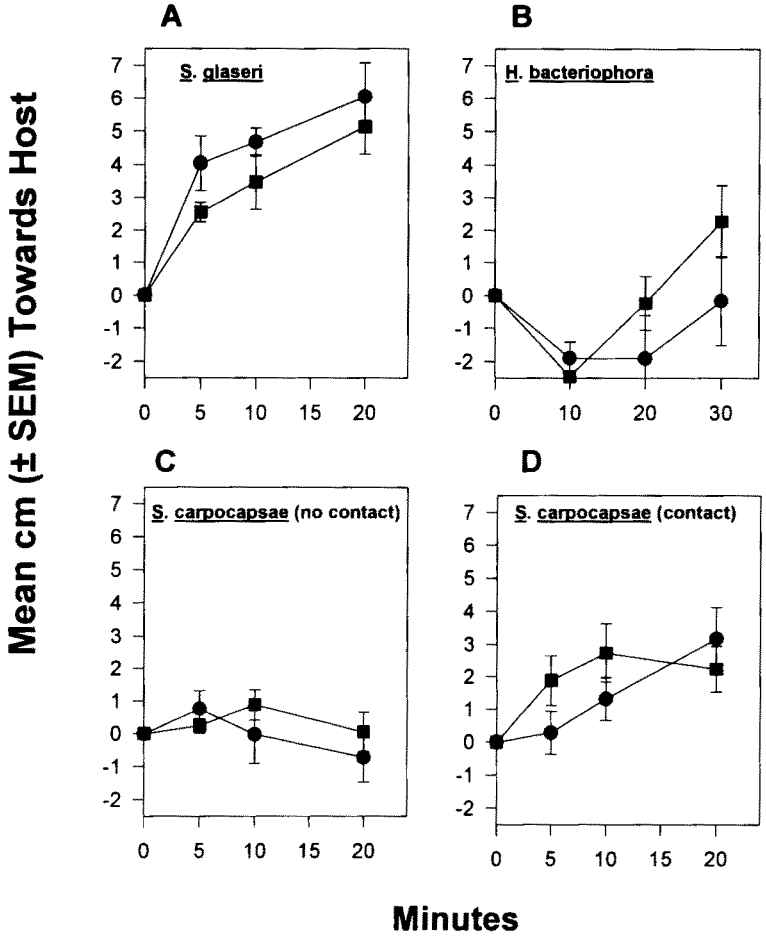

FIG. 4. Effect of wheat germ agglutinin (WGA) treatment of nematodes on movement toward *G. mellonella* volatile cues. (A) *S. glaseri*, (B) *H. bacteriophora*, (C) *S. carpocapsae*, (D) *S. carpocapsae* after exposure to host cuticle. ●, WGA treatment; ■, phosphate-buffered control treatment.

(Jansson and Nordbing-Hertz, 1984). Inhibition of nematode responses to attractants have also been documented for *C. elegans* to *E. coli* filtrates (Jeyaprakash et al., 1985) and male *T. colubriformis* to conspecific female sex pheromone (Bone and Bottjer, 1985). Kerwin et al. (1990) also found that incubation of *Romanomermis culicivorax* with Con A and lentil lectins significantly reduced

nematode attachment to *Anopheles freeborni*. Conversely, lectins have had negligible effects on chemotaxis by *Heterodera schachtii* males in response to female sex pheromone (Aumann et al., 1990) or by *S. carpocapsae* to host plasma (Khlibsuwan et al., 1992). We likewise found that WGA binding had no effect on the response of entomopathogenic nematode infective juveniles to host-produced volatiles on an agar substrate. However, other lectins may yield different results.

Surprisingly, the attachment rate of *S. carpocapsae* infective juveniles was increased by treatment with some lectins. Attachment to passing hosts by this species is primarily passive, so the mechanism for the increase is more likely physical than behavioral. When *G. mellonella* larvae were treated with WGA, attachment of *S. carpocapsae* to the host was significantly decreased from that of the control. This same lectin interfered with the recognition response of *R. culicivorax* to *An. freeborni* when the host was treated with WGA. The decreased attachment to *G. mellonella* suggests lack of recognition on the part of the parasite due to masking by WGA of a specific carbohydrate associated with insect cuticle.

The binding pattern of WGA suggests that nematodes with cruise foraging strategies rely more upon sensory reception of host cues during host location than ambushing nematodes. However, lectin binding to the glycoconjugates associated with their sensory organs had no apparent effect on the response to host cues. Lectins may, however, block behavioral steps leading to parasitization other than host finding in which host volatiles are not involved. Host recognition, in which presumably larger molecules associated with the insect cuticle convey the necessary information, may be affected. Further studies are underway to test the effect of other lectins on host recognition behavior of entomopathogenic nematodes.

Lewis et al. (1993) suggested that species differences in entomopathogenic nematodes' responses to host cues could be explained by the nematodes' foraging strategies. Ambushing parasites would experience little selection pressure to maintain sensitivity to remote cues because they rely upon host movement for host location. We found no specific binding of lectins to the sensory organs of ambushing nematodes, whereas cruising nematodes' amphidial regions were bound by the same lectins. Specific differences in the location of WGA binding sites among nematode species with different foraging strategies may illustrate underlying mechanisms of behavioral differences.

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EFFECTS OF DIET ON LOCALIZED DEFECATION BY NORTHERN PIKE, *Esox lucius*

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Abstract—Fathead minnows (*Pimephales promelas*) are able to detect conspecific alarm pheromone in the feces of northern pike (*Esox lucius*) and have been shown to avoid areas labeled with the feces of pike that were fed minnows. The minnows did not avoid areas labeled with the feces of pike that were fed swordtails (*Xiphophorous helleri*), which lack ostariophysan alarm pheromone. In laboratory experiments, pike fed a diet of minnows localized their defecation away from their foraging area. It has been suggested that in doing so, pike may remove chemical cues that label their foraging area as dangerous to prey species. As yet there has been no conclusive evidence to support this hypothesis. In this experiment, we test the effects of different predator diets on localized defecation by pike. Pike were fed minnows, swordtails, or mice (*Mus musculus*). Swordtails and mice lack ostariophysan alarm pheromones. Area use and location of feces were recorded. Pike fed minnows spent significantly more time in the home area (i.e., area of the test tank where they were fed) and defecated significantly more often in the opposite end of the tank. Pike fed swordtails also exhibited a significant preference for the home area in area use, while those fed mice showed no such preference. When fed either swordtails or mice, there was no significant difference between the proportion of time spent and proportion of feces in each area of the test tank. These data suggest that pike localize their defecation only when consuming prey items containing alarm pheromone. The current findings support the hypothesis that pike localize their defecation to remove chemical cues from the foraging area of the home range in order to avoid chemically labeling their foraging area as dangerous to prey.

Key Words—Fathead minnow, *Pimephales promelas*, northern pike, *Esox lucius*, localized defecation, predator labelling, alarm substance.

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INTRODUCTION

Fathead minnows (*Pimephales promelas*) possess epidermal club cells, which, when mechanically damaged, release an alarm pheromone [alarm substance (AS) or Schreckstoff (Smith, 1986, 1992)]. Mechanical damage typically occurs when a minnow is attacked or captured by a predator. When detected by conspecifics, AS elicits a stereotypic fright response, characterized by a variety of antipredator behavior patterns (Heczko and Seghers, 1981; Lawrence and Smith, 1989; Krause, 1993; Mathis and Smith, 1993a). Mathis et al. (1995) have demonstrated that predatory northern pike (*Esox lucius*) are attracted to minnow alarm pheromone.

Mathis and Smith (1993a,b) have recently demonstrated that fathead minnows that had never encountered a pike responded with antipredator behavior to chemical stimuli (water containing urine, feces, mucus that had housed a pike) from pike if the pike had recently consumed minnows, but not swordtails (*Xiphophorus helleri*) or breeding male minnows. Swordtails lack ostariophysan alarm pheromones (Mathis and Smith, 1993a,b), and breeding male fathead minnows lose their alarm substance cells during the spawning season (Smith, 1973, 1976). Brown et al. (1995a,b) have demonstrated that AS is contained in feces of a predatory pike and that minnows that have never encountered a pike will avoid areas labeled with feces of pike that have been fed minnows but do not avoid feces of pike fed swordtails.

Brown et al. (1995a) showed that pike fed fathead minnows localize their defecation away from their foraging area. They suggested that pike may do so in order to remove chemical cues that could label their foraging territory as dangerous to prey fish. As yet, there has been no conclusive evidence to support this hypothesis. In the current study, we examine the effects of different diets on localized defecation by pike. We predict that if pike are localizing their defecation to remove possible chemical cues from their foraging range, then pike fed a diet which lacks AS should not show localized defecation.

METHODS AND MATERIALS

Test Fish. Northern pike were collected with seine nets from Van Patten's Creek in south central Saskatchewan. Pike were transported to the lab and held in 60-liter holding tanks at approximately 16°C, under a 14 : 10 light–dark cycle. Prior to the experiments, pike were fed one or two fathead minnows once every four days.

Fathead minnows were taken from Pike Lake, an oxbow lake of the South Saskatchewan River near Saskatoon, Saskatchewan. Minnows were held in 300-liter artificial stream channels under a 14 : 10 light–dark cycle. Minnows were

fed daily with commercial fish food. Swordtails (maintained on same diet as minnows) and frozen neonatal mice (*Mus musculus*) were obtained commercially. Swordtails were used as a fish prey because they lack ostariophysan alarm pheromone (Mathis and Smith, 1993a). It is unlikely that swordtails possess any form of alarm pheromone, as two additional species of *Xiphophorus* (*X. maculatus* and *X. variatus*) have been shown to lack alarm pheromones (Pfieffer, 1977). Mice were chosen as a representative terrestrial prey because they are taken opportunistically by inshore pike (Lawler, 1965; Scott and Crossman, 1973) and lack ostariophysan alarm pheromone. Mice possess disturbance chemical alarm signals, which are released in urine and/or feces when disturbed (Rottman and Snowdon, 1972). These cues are likely not present in our trials, since the mice were dead when fed to the pike. In addition, under natural conditions, mice would be swimming near the surface, while feces would be restricted to the substratum. Mice were thawed in water prior to use.

Test Tanks. Three, identical 500-liter glass aquaria (183 cm long × 49 cm wide × 56 cm high) were used as test tanks. The test tanks were not filtered, and there was no appreciable flow. A single airstone was suspended near the back wall of the tank, halfway down the length of the tank. We divided each tank into seven compartments, as in Brown et al. (1995a), by drawing gridlines on the exterior of the tank. The first compartment was 38 cm wide, and each of the remaining six compartments were 24 cm wide. The first compartment was designated as the home area and contained rocks and artificial plants that extended to within 15 cm of the water surface. In addition, opaque plastic covers were placed over the home range to provide shade. The floor of the entire tank was covered with silica sand to a depth of 4 cm. Water temperature in the testing tanks was maintained at approximately 18°C. Test tanks were illuminated with overhead fluorescent lights and maintained on a 14:10 light–dark cycle.

Experimental Protocol. Trials lasted one week, and each trial consisted of five days of acclimation and two days of testing (days 6 and 7). An individual pike was placed in the test tank on the morning of day 1. During days 1–5, we fed each pike one of the following diets: fathead minnow, swordtail, or mouse. For each condition, pike were fed one food item daily (mean ± 1 SD = 1.45 ± 0.21, 1.82 ± 0.32, and 1.71 ± 0.27 ml, minnow, swordtail, and mouse, respectively, measured by volumetric displacement in water). Prior to feeding, minnows and swordtails were killed by a blow to the head. Each food item was injected with approximately 0.5 ml of ultraviolet fluorescent dye (Tracer Glo). Since pike do not readily accept dead prey (Huntingford, 1984), prey items were suspended on the end of a fine-gauge stainless steel wire. The use of the wire allowed us to manipulate the prey item and induce the pike to strike. In the case of minnow or swordtail diets, the wire was placed at the base of the caudal peduncle. In the case of the mouse diet, the wire was placed through a fold of

skin near the hind legs. This allowed us to pull the wire free without disturbing the pike. Prey were always presented in the home area.

At the end of day 5, all feces were removed from the test tank with a siphon. On day 6, we began recording two measures: (1) proportion of time the pike spent in each area of the tank (area use), and (2) the location of feces. We quantified area use by recording the location of each pike once every 10 sec for a 10-min observation period once each hour for eight consecutive hours on days 6 and 7. Observations began at approximately 08:00 hr each day.

The location of feces was quantified after the final area use observation on days 6 and 7. We recorded (and removed with a siphon) the number of fecal pellets in each compartment of the test tank. Fecal pellets within 1 cm of each other were recorded as a single pellet. After recording the location of feces on day 7, we removed the pike and carefully drained the tank so as not to disturb the substrate. A UV light was used to check for any fecal pellets that may have partially dissolved or been covered with sand. We recorded the presence of fluorescent dye as a fecal pellet, using the same criterion as above.

A total of nine pike (mean $\pm$ SD = 18.21 $\pm$ 2.61 cm) were used in the experiment. Each pike was tested once in each diet condition. The order of trials was random, and pike were tested once every three weeks. Individual pike were tested in different tanks for each of the three diet conditions. No qualitative differences were observed in the acceptability of each of the three prey types.

Statistical Analysis. We employed a one-way analysis of variance (ANOVA; Sokal and Rohlf, 1981) to determine if there was a significant difference in the amount of time spent or the number of fecal pellets in each of the seven areas of the test tank. We compared the percent of time versus the percent of total feces in each area of the tank using paired t tests, corrected for increasing alpha using a modified Bonferronni procedure (Keppel 1982).

RESULTS

Pike fed minnows and pike fed swordtails spent a significantly greater proportion of time in the home area end of the test tank ($F_{6,56} = 13.22$, $P < 0.0001$ minnow diet, $F_{6,56} = 3.90$, $P < 0.003$ swordtail diet; Figure 1). Pike fed mouse did not localize their time in any section of the test tank ($F_{6,56} = 1.21$, $P = 0.30$; Figure 1). In the minnow diet trials, pike defecated significantly more often in the opposite end of the test tank ($F_{6,56} = 54.30$, $P < 0.0001$; Figure 1). Pike fed swordtails defecated significantly more often in or near the home area ($F_{6,56} = 3.90$, $P < 0.003$; Figure 1). In the trials of pike fed mouse, there was no significant difference in the number of fecal pellets in any of the seven areas of the test tank ($F_{6,56} = 1.21$, $P = 0.32$; Figure 1).

When pike were fed minnows, there was a significantly greater proportion of time spent in the home area and the two adjacent areas compared to the

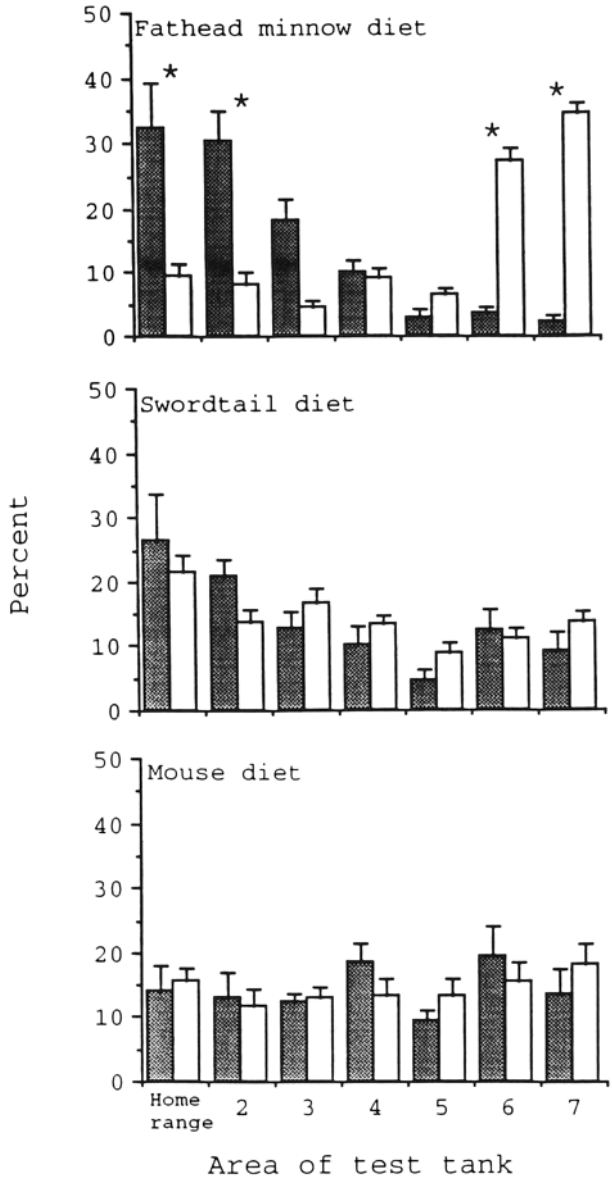

FIG. 1. Mean (±SE) percent time spent (closed bars) and percent total feces (open bars) in each area of the test tank for each of the three diet conditions. An asterisk denotes significant difference ($P < 0.05$) for comparison between percent time and percent feces in each area of the test tank. See text for details.

percent of total feces ($P < 0.05$, Figure 1). Conversely, there was a significantly greater proportion of feces compared to the percent of time in areas 6 and 7 of the test tank (Figure 1) when fed minnows. In both the swordtail and mouse diet conditions, there was no significant difference in the percent time vs. percent feces in any area of the test tank (Figure 1).

DISCUSSION

The results of the current study clearly demonstrate an effect of diet on localized defecation by northern pike. When fed a diet of fathead minnows, pike localized their defecation away from the home or foraging area, confirming the results of Brown et al. (1995a). When fed a diet of either swordtails or mice, which lack alarm pheromones, there was no difference between the percent of time spent and percent of total feces in each area of the test tank, indicating that the pike did not localize their defecation when fed these diets. These results support the hypothesis that pike localize defecation in order to avoid chemically labeling their foraging area as dangerous to potential prey.

There exist alternate hypotheses that may explain the observed localized defecation in pike. In particular, pike may localize defecation for sanitary reasons. By defecating away from the home or foraging range, pike may decrease the likelihood of pathogen or parasite infection. Such a system has been shown for a variety of avian and mammalian species (Poole, 1985). While the current data cannot refute the antipathogen/parasite hypothesis, we observed localized defecation only in response to the minnow diet. Our data do support the hypothesis that pike localized defecation to remove chemical cues from their foraging territory. In addition, these hypotheses are not mutually exclusive and could both serve as selection pressures for localized defecation behavior in pike.

These data demonstrate an effect of diet on the foraging patterns of pike. In both the minnow and swordtail diet conditions, pike spent a significantly greater proportion of time in the home end of the test tank, while in the mouse diet condition, pike did not exhibit such a preference. Since pike are primarily ambush predators (Hobson 1979; Chapman and MacKay, 1984; Savino and Stein, 1989), it would benefit a pike to remain under the cover of vegetation and wait for suitable prey to come within its attack range. Conversely, terrestrial prey items, such as mice, would be swimming near the surface. Hence, when fed a mouse diet, it would benefit the pike to remain in open water. These differences in foraging strategy would account for the lack of localized area use observed in the mouse diet condition, even though the pike were fed in the same manner regardless of diet condition.

These results also indicate a more general phenomenon, whereby the foraging strategy of predators is affected by diet. By altering the foraging strategy

to match the most abundant prey type, the pike would maximize its probability of future foraging success. As a result, if pike have experienced terrestrial prey in open water and are subsequently fed terrestrial prey, then we would predict that they should not show a preference for area of the test tank containing vegetation.

There are a variety of mechanisms by which the presence of alarm pheromone in the home or foraging area could decrease a pike's foraging efficiency. Initially, Brown et al. (1995a) demonstrated that the alarm pheromone is present in the pike's feces. The presence of feces containing minnow alarm pheromone has been shown to decrease area use by minnows (Brown et al., 1995b). This would serve to decrease the pike's foraging efficiency by deterring prey fish from entering the pike's foraging area.

Chivers and Smith (1995a) have shown that fathead minnows are able to learn the identity of risky habitats when the chemical cues of a particular microhabitat are paired with AS. If AS in pike feces were present in the pike's foraging range, then the minnows may learn to avoid the risky area. Alternatively, the minnows may be more vigilant when in the area and therefore less likely to be captured (Mathis and Smith, 1993c; Chivers and Smith, 1995a). Given that minnows can culturally transmit recognition of dangerous habitats (Chivers and Smith, 1995b), the effects may extend to other minnows.

Cross-species reactions to alarm pheromones have been identified in a variety of species (e.g., Smith, 1982; Smith et al., 1991; Mathis and Smith, 1993d; Chivers and Smith, 1994; Wisenden et al., 1994, 1995; Chivers et al., 1995a,b). Prey species typically avoid areas containing heterospecific alarm pheromones of members of their prey guild (i.e., those species with which they share predators). The presence of minnow alarm pheromone in the pike's foraging range would likely also decrease area use by other species, such as brook sticklebacks (Mathis and Smith, 1993d), that recognize and respond to minnow AS. By defecating away from their foraging range, pike can increase the likelihood of future predation events in that location not only on minnows, but on other species of the prey guild as well.

Minnow alarm pheromone has been shown to attract pike (Mathis et al., 1995). A pike that is attracted to AS (i.e., a secondary predator) could interfere with a primary predator that has captured a minnow. The secondary pike could compete for the prey item directly, or it could attack the primary predator as a prey item. Avoidance of secondary predator interference could account for the observed localized defecation. By removing minnow alarm pheromone from their foraging range, pike could decrease the possibility of attracting secondary predators.

In summary, the data presented in the current study demonstrate an effect of diet on the localized defecation of northern pike. Localized defecation was observed when pike had consumed minnows, but not when pike had been fed

swordtails or mice. These data thus support the hypothesis that pike localized their defecation in order to avoid chemically labeling their foraging area with the alarm pheromone of their prey.

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SEASONAL CHANGE IN NAGILACTONE CONTENTS IN LEAVES IN *Podocarpus nagi* FOREST

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Abstract—Nagilactones isolated from *Podocarpus nagi* (Thunb.) Zoll. et Moritz. are known by their physiological activities as a plant growth inhibitor or antiherbivory substance. As the first step in clarifying the nagilactone dynamics in a forest canopy, the seasonal variations in nagilactone contents in leaves and mass of nagilactones accumulated in the canopy were examined at Mt. Mikasa, Nara City, central Japan. Nagilactone content in mature leaves dropped sharply in May, when new leaves flushed. The nagilactone content of new leaves was far greater than that of mature leaves, implying a translocation of nagilactones from old leaves to new leaves. The total mass of nagilactones in the canopy was 3.9 kg/ha.

Key Words—*Podocarpus nagi*, nagilactone, deer, antiherbivory, forest canopy.

INTRODUCTION

Nagilactone isolated from *Podocarpus nagi* (Thunb.) Zoll. et Moritz. (Ohwi, 1965) by Hayashi et al. (1968, 1972) has six structural isomers. Its physiological activities as a plant growth inhibitor are well known as described elsewhere (Hayashi and Sakan, 1974; Hayashi et al., 1975, 1979; Ishikawa, 1971; Suganuma, 1975). A mixture of nagilactone and other chemicals, such as epicatechin, may reduce the original inhibitory activity of nagilactone (Kubo et al., 1985). Furthermore, nagilactone is an antiherbivory chemical for animals, such as insects and deer (Hayashi and Sakan, 1974), and has antitumor activity for

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sarcoma (Hayashi et al., 1979). However, the effects of nagilactone on plant growth and sustainment of *P. nagi* populations in a natural forest still remain obscure, although the negative effects of nagilactone on trees other than *P. nagi* in the forest have been suggested by ecological studies (Koshimizu, 1943; Suganuma, 1975; Tamai, 1989). As the first step in clarifying the nagilactone dynamics, in situ, the present study dealt with seasonal change of nagilactone concentrations in leaves.

METHODS AND MATERIALS

Study Forest. Our study was carried out in a *Podocarpus nagi* forest, on a western slope of a low hill, Mt. Mikasa, 294 m alt., 40 ha area, 34 N latitude, and 135 E longitude, in Nara City, central Japan (Figure 1). The forest is part of a forest of the Kasuga Shinto Shrine, which was established in the fifth century. In this forest, two forest types have been identified. One is the *P. nagi* forest and the other is a warm-temperate evergreen rain forest with oak and laurel dominants. The latter is the original vegetation around Mt. Mikasa

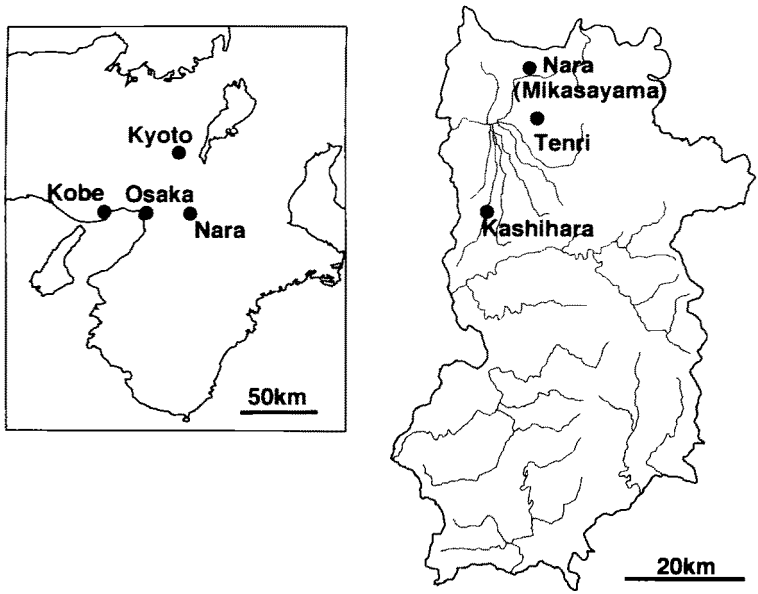

FIG. 1. Map of the Kansai area and Nara Prefecture, showing study site, Mt. Mikasa, in the northern Nara Prefecture.

(Suganuma, 1975; Miyawaki, 1984) and is tentatively designated the mixed oak–laurel forest.

The *P. nagi* forest is believed to be derived from a few pairs of female and male trees transplanted to this site from another Shinto shrine, since *P. nagi* is dioecious and has never been recorded in native vegetation around Nara City (Koshimizu, 1943; Suganuma and Kawai, 1978). The *P. nagi* forest dominates the original mixed oak–laurel forest through competition between *P. nagi* and other trees. The forest has been protected without any human manipulation of the vegetation.

Deer Protected by Kasuga Shinto Shrine. Deer, *Cervus nippon,* have also been protected by the Kasuga Shinto Shrine, since they were believed to be holy animals by the Shinto followers. According to the Society of Deer Conservation, Nara (1994), the population density of deer might be rather constant, about 1000 head in an area around the forest, although it may have decreased in socially unstable eras, such as war time. Recent records of the deer population suggested a rather stable population density after a recovery from a disaster at the time of the Second World War (Figure 2). The deer feed freely in and out of the forest and have been influential in the structure and composition of the forest (Suganuma, 1975; Shimoda et al., 1994).

Climate Conditions. The climatic data recorded from 1961 to 1990 at the Nara Meteorological Station (104 m alt.) about 2.5 km northwest of our study forest at Mt. Mikasa indicates a rather mild climate at the study site. Mean annual temperature was 14.4°C with a range of mean monthly temperatures of −0.7°C (January) to 22.2°C (August). Mean annual precipitation was 1355 mm, with a mean monthly rainfall range of 40.4 mm (December) to 212.3 mm (June). Frost was common in winter, but snow was seldom over 10 cm.

Study Plot and Forest Inventory. A research plot of 30 × 30 m was established on a hill slope dominated by *P. nagi* at Mt. Mikasa in May 1991. The

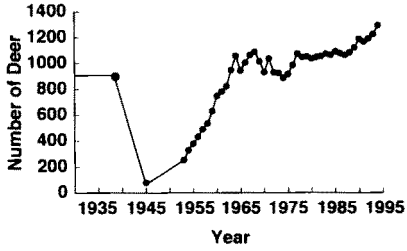

FIG. 2. Population density of deer protected by the Kasuga Shinto Shrine. Data were supplied by the Society of Deer Conservation, Nara. The population density decreased during World War II.

plot was selected so as to represent a mature phase of the forest growth cycle (Whitmore, 1984). The plot was subdivided into 7.5 × 7.5-m quadrants following conventional procedures for a forest inventory. In each quadrant, all trees and woody climbers 1.0 cm and more in stem diameter at breast height (DBH, 1.3 m above the ground) were individually labeled, mapped, identified, and measured (Figure 3).

Leaf Biomass. Leaf biomass in the study plot was estimated by summing up the leaf mass of individual trees by using the allometry method (Ogawa, 1977). Various dimensions and partial biomasses of trees were calculated from DBH (D, centimeters) by allometric equations for estimating individual tree leaf mass. The calculated quantities were tree height H (meters), stem dry weight $W(S)$ (kilograms), branch dry weight $W(B)$ (kilograms), and leaf dry weight $W(L)$ (kilograms) per tree. We adopted the following allometric equations proposed by Wang (1993):

$$1/H = 1/(1.41D) + 1/31.34$$

$$W(S) = 0.0394\ (D^2H)^{0.9187} + 0.120$$

$$W(B) = 0.140\ [W(S)]^{1.031}$$

$$W(L) = 0.173\ [W(B)]^{0.699}$$

Various trees, such as oaks, laurels, etc., were recognized in lower forest layers. To estimate the leaf biomass of flowering trees other than podocarps, we used the following allometric relations (Ogawa and Saito, 1965):

$$1/H = 1/(2.12D) + 1/10.4$$

$$W(S) = 0.0396\ (D^2H)^{0.9326}$$

$$W(B) = 0.0602\ (D^2H)^{1.027}$$

$$1/W(L) = 21.6/[W(S) + W(B)] + 0.02$$

Among these allometric equations for flowering trees, the relation between tree height, H, and stem diameter, D, were obtained by the forest inventory in this study. The other allometric equations were obtained by cutting sample trees (Ogawa and Saito, 1965).

Green Leaf Sampling. Green podocarp leaves were sampled monthly from October 1991 to October 1992 for chemical analysis of nagilactone contents in living leaves. A large female tree (27.8 cm DBH and 20.1 m tree height) was selected for the sampling around the plot for the forest inventory (Figure 3). The sample leaves were grown in sun, sclerophyllous, fully matured, and 2 years old. New leaves were separately sampled on June 22 to compare their nagilactone contents with those of mature leaves.

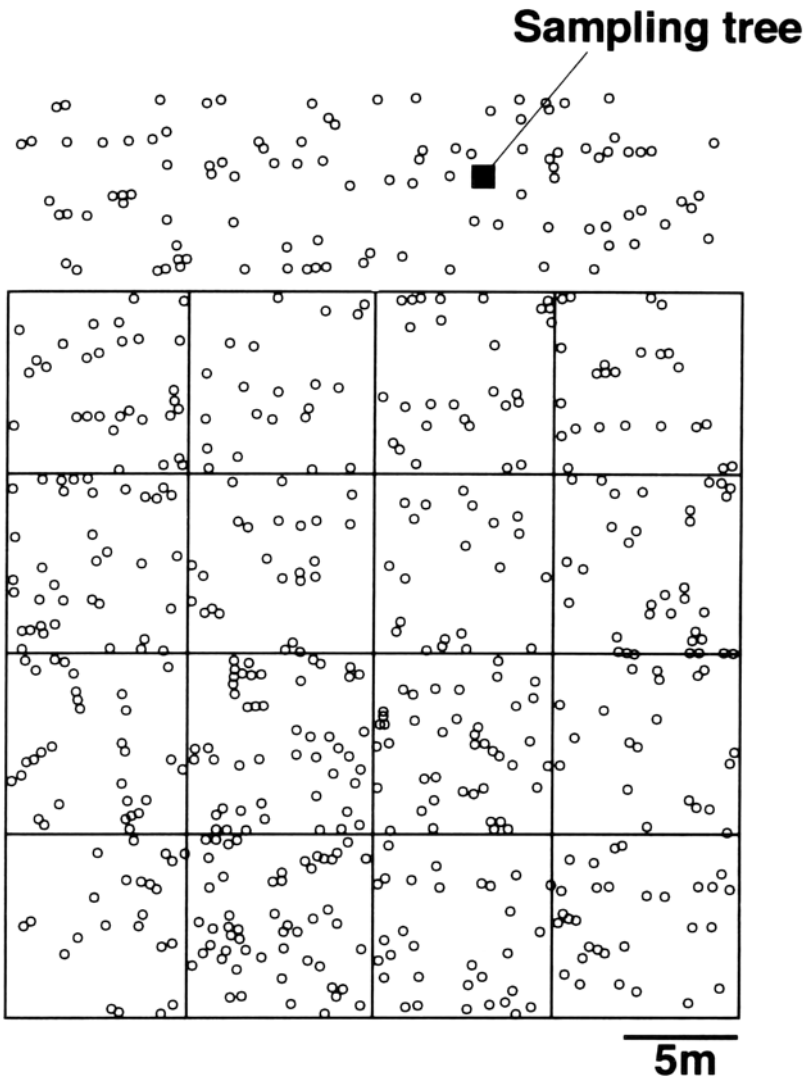

FIG. 3. Map showing positions of individual podocarp trees in the study plot. Circles indicate tree positions; the solid square represents the position of a sample tree for the determination of leaf nagilactone contents.

Nagilactone. Six structural isomers of nagilactones have been identified by Hayashi (1973), as already described. They are nagilactones A ($C_{19}H_{24}O_6$), B ($C_{19}H_{24}O_9$), C ($C_{19}H_{24}O_8$), D ($C_{18}H_{20}O_8$), E ($C_{19}H_{24}O_6$), and F ($C_{19}H_{24}O_4$) (see Figure 4). Furthermore, they are characterized by different melting points, 305, 258–261, 290, 265–266, 295, and 225–226°C, respectively (Hayashi, 1973; Singh et al., 1979). Among these isomers, nagilactones A, B, C, and D are contained in leaves, while nagilactones E and F are found in the roots (Hayashi, 1973). Pure nagilactones were supplied by Hayashi for standard materials in this study.

Extraction of Nagilactone from Leaves. Podocarp leaves were first crushed in a blender for easy extraction of nagilactones. Crushed leaves were kept in pure methanol in glass bottles for two weeks. A mixture of various substances extracted from leaves with methanol was vacuum-filtered through a Buchner funnel using Advantec No. 5A filter paper. The filtered aqueous fraction was evaporated at 40°C, to remove methanol. The evaporated fraction was successively partitioned with hexane, dichloromethane, and ethyl acetate. These three organic fractions were dehydrated with anhydrous magnesium sulfate, filtered through a Buchner funnel, evaporated in a rotary evaporator to dryness at 40°C, and the solid residue weighed. The three crude solids were kept in a refrigerator at −30°C.

HPLC Analysis. A crude solid was dissolved with methanol for HPLC analysis. If the weight of the solid was 1.0 mg, 1.0 ml methanol was used for

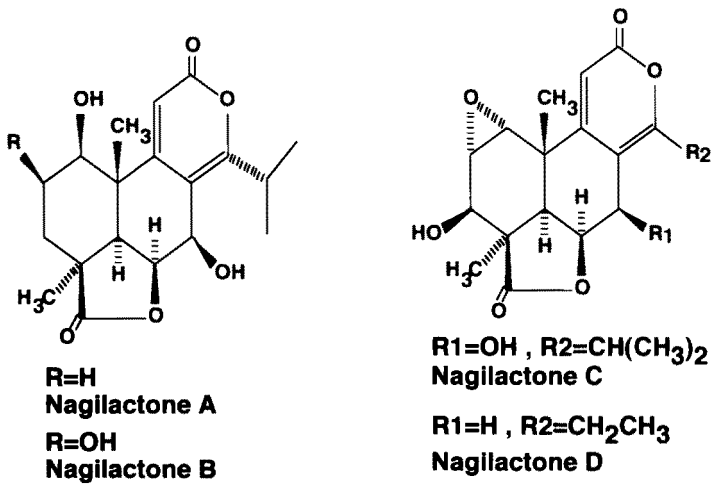

FIG. 4. Chemical structure of nagilactones.

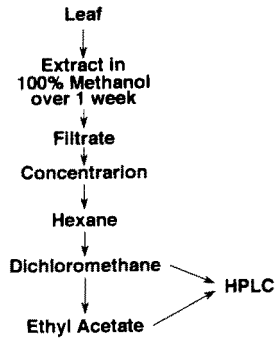

FIG. 5. Summary of procedures for fractionation of nagilactones.

solvent. The mixture of nagilactones dissolved in methanol was separated into respective nagilactones by a Waters HPLC apparatus on a Shiseido Capcell Pak C18 cartridge column (S-5 μl 4.6 $\times$ 250 mm), employing water–acetone–methanol (7:2:1). Nagilactones A, C, and D were found, while the other nagilactones were not observed in samples. Nagilactone B was rarely reported in leaves, and nagilactones E and F were mainly contained in root bark (Hayashi and Sakan, 1974). Separated nagilactones A, C, and D were quantified by a Waters variable-wavelength UV spectrophotometer at 300 nm wavelength (Figure 5).

RESULTS

Stand Structure. In addition to 137 podocarp trees, only three flowering tree species were observed in our 30 $\times$ 30-m research plot. These were *Neolitsea aciculata* (88 individuals), *Pieris japonica* (three individuals), and *Symplocos prunifolia* (one individual). The former two species have unpalatable leaves, which contain toxic secondary metabolites, such as dodecanoic acid in *N. aciculata* (Glasby, 1991) and asebotoxins in *P. japonica* (Glasby, 1991), and are seldom eaten by deer or other animals. The latter species, *S. prunifolia,* seemed to be an escape from, or remnant of, the original vegetation, mixed-oak-laurel forest, which covers the entire 250 ha at the Kasugayama hill about 500 m to the east of the study plot. *S. prunifolia* is a common pioneer tree whose seeds are dispersed by small birds in the mixed oak–laurel forest. In the forest at Kasugayama, about 500 m east of our study plot, 20 or 30 tree species are easily recognizable in a small area (Fujii, 1991), such as in the 30 $\times$ 30 m plot demarcation in this study. Thus, the species diversity of the study forest was the lowest among the natural forest vegetations at lower altitudes in Japan,

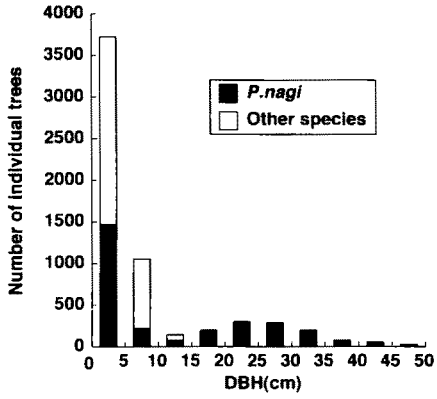

FIG. 6. Frequency distribution of DBHs of individual trees in the study plot.

suggesting strong effects of toxic secondary metabolites of plants on stand architecture.

The frequency distribution of DBHs of individual trees within the plot (Figure 6) represents high densities of smaller podocarp and *N. aciculata* trees, respectively, suggesting continuous germination, good seedling growth, and perpetual self-maintenance of the two species. It is noteworthy that *N. aciculata* does not grow larger than podocarps, but they formed the dense second story beneath the first story of podocarps. Thus, the study forest looked like a pure stand of podocarps in its physiognomy.

Leaf Biomass. The leaf mass of individual trees in the stand was calculated by the allometry method already described. The results are summarized in Figure 7, which represents the frequency distribution of the leaf mass of individual trees in the stand. Leaf biomass estimates were 10.2 ton/ha and 0.5 ton/ha for podocarps and the other three species, respectively. The total leaf biomass of 10.7 ton/ha resembled leaf biomass in broad-leaved evergreen rain forests in Japan, and the shape of podocarp leaves is similar to that of broad-leaved evergreen trees. These values are necessary for calculating the total amount of nagilactones accumulated in the forest canopy, as described later.

Nagilactone Calibration Lines. The retention times of nagilactones A, C, and D on HPLC were 10.6, 7.6, and 13.0 min, respectively. Denoting the height of a peak at each retention time by h (in centimeters), the calibration lines for three nagilactone contents in samples were approximated by: $C(A) = 0.0638h$, ($r^2 = 0.99$), $C(C) = 0.0564h$, ($r^2 = 0.99$), and $C(D) = 0.0921h$, ($r^2 = 0.99$), where $C(A)$, $C(C)$, and $C(D)$ stand for the concentrations of three nagilactones A, C, and D, respectively.

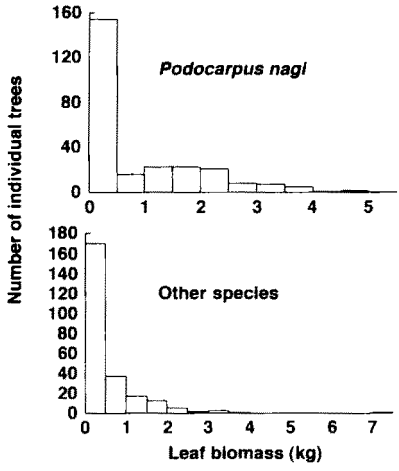

FIG. 7. Frequency distribution of individual leaf mass in dry weight within the study plot.

Nagilactone in Leaves. Nagilactone content in mature green leaves was quantified by HPLC techniques and calibration lines. Nagilactone content was expressed as the amount of nagilactone per unit leaf dry weight, and biomass was expressed by the unit of dry weight. Nagilactone C was the highest in concentration among the three isomers throughout a year (Figure 8). Nagilactones A and D were not detected in some samples. Their concentrations apparently fluctuated seasonally (Figure 8). The total nagilactone concentration per unit leaf dry weight increased in the winter season from December to March. The highest peak of 777.2 μg/g in nagilactone concentration in March sharply dropped to 83.6 μg/g in May (Figure 8). This sudden drop in nagilactone concentration in old leaves corresponded to a high concentration of 972.2 μg/g (sum of nagilactones A, C, and D) in new leaves sampled in June 1992. The concentration in new leaves was far greater than that (472.5 μg/g) in mature leaves sampled in June 1992.

The mass of nagilactones accumulated in canopy leaves is easily computed by multiplying the leaf biomass (10.2 ton/ha) of podocarps by nagilactone contents per unit leaf weight. For purposes of computation, a mean nagilactone content per year was estimated to be 2.91×10^{-5} g/g for nagilactone A, 2.94×10^{-4} g/g for nagilactone C, and 6.10×10^{-5} g/g for nagilactone D. The nagilactone mass values thus calculated were 296.8 g/ha, 2,998.8 g/ha, and 622.2 g/ha for nagilactones A, C, and D, respectively. The total of these values was 3,917.8 g/ha.

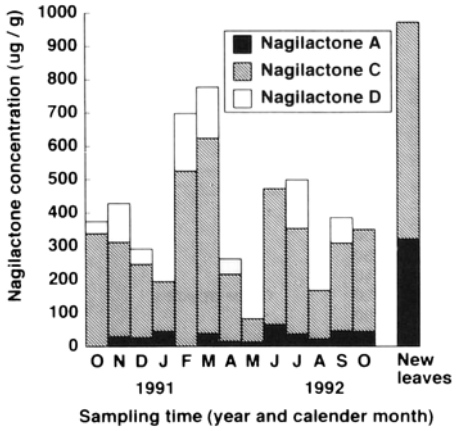

FIG. 8. Seasonal change in nagilactone content in leaves. A large nagilactone content in new leaves, 972.2 μg/g, was recorded in June 1992.

DISCUSSION

The dominance of *P. nagi* in our study forest is clear, since 95% of canopy leaves consisted of podocarp leaves in terms of leaf biomass. The mono-dominance of podocarps in forest stands is rare in Japan, as described below.

Trees of the genus *Podocarpus* are subcosmopolitan conifers, which are distributed mostly in the Southern Hemisphere but extend into the Northern Hemisphere crossing a tropical life zone (Willis, 1973). Among 100 species of the genus *Podocarpus*, two species and one variety are recognizable in Japan, which demarcates the northern latitudinal boundary in the natural geographical range of podocarp trees. Two of the Japanese podocarps, (*P. nagi* Thunb, and *P. macrophyllus* Siebold) are tall and native to Japan, while the other (*P. macrophyllus* var. Maki Siebold) is a dwarf and was introduced from China (Kitamura and Murata, 1979). The former two are mostly found as scattered individuals and their tree density is low, intermingled with various trees in the mixed oak–laurel forests. The two podocarps are generally rare in original Japanese forests (Suganuma, 1975), and the dense growth of *P. nagi* in our study site at Mt. Mikasa is unusual.

According to legend some *P. nagi* were transplanted in the forest with oak–laurel dominants by a Shinto follower to soothe the Shinto spirits over 1000 years ago (e.g., Koshimizu, 1943). If that is true, the podocarp forest at our study site offers a typical example of ecological invasion. Japanese botanists

and ecologists have suggested the following hypotheses, reviewed by Koshimizu (1943) and Suganuma (1975) to account for the dense growth of *P. nagi* at Mt. Mikasa: (1) an appropriate sex ratio of transplanted founders, (2) good soil conditions for podocarp growth at Mt. Mikasa, (3) the high shade tolerance of podocarp seedlings, (4) inhibitory effects of nagilactone on the growth of other plants, and (5) the unpalatability of podocarp leaves resulting in the escape from feeding pressures of herbivores, such as deer protected by the Shinto Shrine. The fourth suggestion stresses the importance of the allelopathy of podocarps, and the last hypothesis is related to the biological activity of nagilactone as an antiherbivory substance. The results of the present study are related to the last hypothesis.

The total nagilactone contents in mature leaves changed from month to month and their annual mean was 3.84×10^{-4} g/g (Figure 8). By multiplying this value and the weight of a single leaf, 0.17 g in dry weight, we get the mean mass of the total nagilactone, 6.53×10^{-5} g, contained in a leaf. If this quantity of nagilactone could dissolve in leaf water, 0.184 g per leaf on average, its liquid concentration per leaf would be equivalent to 1×10^{-3} mol/liter in terms of the largest molecular weight of nagilactone C. This concentration may be enough to inhibit plant growth, since the nagilactone concentrations for 50% growth inhibition for *Avena* coleoptiles were much less than 10^{-4} mol/liter (Hayashi and Sakan, 1973). A nagilactone concentration of 1×10^{-3} mol/liter may be available for defense against insects and vertebrates. Grazing scars were not found on living leaves and dead leaves in our experience. Repeated feeding tests of podocarp leaves to deer showed that deer never eat podocarp leaves.

The seasonal change in nagilactone concentrations in leaves suggested the possibility of translocation of nagilactone from old leaves to new leaves. Alternatively, new leaves might actively produce nagilactone. To confirm the translocation of nagilactone in terms of production ecology, the daily changes in photosynthesis, leaf respiration, growth, and nagilactone content of new leaves must be measured simultaneously. The high concentration of nagilactone in new leaves may protect them from herbivores.

The poor species composition of the podocarp forest, consisting of a few poisonous trees, suggested an important role of deer browsing in the maintenance of the forest. This fact coincided well with the biological activity of nagilactone, such as a growth inhibitor or antiherbivory measure and supported the aforementioned last hypothesis.

The total mass of nagilactones accumulated in canopy leaves was 3.9 kg/ha, and this provides a basis for evaluating the flow of nagilactone in a forest ecosystem through litter fall and throughfall. Nagilactone in leaves was 3.84×10^{-4} g/g annual mean content and probably worked effectively against the feeding pressure by animals.

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FATE OF THE CHROMENE ENCECALIN IN THE INTERACTION OF *Encelia farinosa* AND ITS SPECIALIZED HERBIVORE *Trirhabda geminata*

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Abstract—Leaf beetles of the species *Trirhabda geminata* are specialized herbivores that are able to feed on the chemically well-protected foliage of the desert sunflower *Encelia farinosa*, which contains the insecticidal chromene derivative encecalin. Chemical analysis of the beetles and their fecal excretions indicated that encecalin is present only in the alimentary canal and is not absorbed across the gut membrane, as previously shown for other herbivorous insects (e.g., the Egyptian armyworm, *Spodoptera littoralis*) that are susceptible to this chromene derivative. Further differences between *T. geminata* and nonadapted insects were observed with regard to the metabolism of encecalin. Whereas the encecalin-resistent leaf beetles metabolize encecalin mainly to encecalol by reduction of the acetyl group, susceptible insects, such as larvae of *S. littoralis*, metabolize encecalin mainly by epoxidation of the 3,4 double bond, which creates a powerful alkylating agent and is responsible for the toxicity of encecalin. Reductive rather than oxidative metabolism of encecalin therefore seems important for the resistance of *T. geminata* against the chemical defense of their host plant *E. farinosa*.

Key Words—*Trirhabda geminata*, Chrysomelidae, *Encelia farinosa*, Asteraceae, chromenes, benzofurans, chemical defense, plant–insect interactions.

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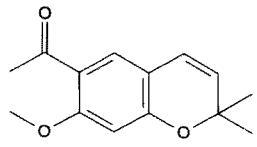
INTRODUCTION

Chromene derivatives (e.g., **1,** Figure 1) and the structurally related benzofurans (e.g. **8,** Figure 1) occur frequently in species of the Asteraceae (Proksch and Rodriguez, 1983; Proksch and Kunze, 1995). In species of the desert sunflower *Encelia* Adans. (Asteraceae) chromenes are detected in all plant parts analyzed, including roots, stems, leaves, and flowering heads. The highest concentrations (on a dry weight basis) of these natural products, however, are usually found in soft and vulnerable tissues such as young expanding leaves or in flowering heads (Proksch and Rodriguez, 1984), where up to 250 μmol chromenes/g dry wt may be present (Kunze et al., 1995). These valuable and important plant parts are thought to be chemically defended by chromenes such as encecalin (**1,** Figure 1) against herbivorous insects (Kunze et al., 1995). The insecticidal activity of encecalin and of related naturally occurring chromenes has, in fact, been demonstrated repeatedly. Insects that proved susceptible to encecalin or related compounds included the migratory grasshopper, *Melanoplus sanguinipes* (Isman et al., 1986); the variegated cutworm, *Peridroma saucia* (Isman et al., 1987b); and the Egyptian armyworm, *Spodoptera littoralis* (Srivastava and Proksch, 1991).

In spite of the potent insecticidal activity of encecalin and several of its derivatives, larvae and adults of the leaf-feeding herbivore, *Trirhabda geminata* Horn (Chrysomelidae), are able to feed exclusively on *E. farinosa* and constitute the primary insect herbivore on this species (Hogue, 1970). Whereas the tolerance of these beetles towards the defensive chemistry of *E. farinosa* has been amply demonstrated in the past (Wisdom, 1985), neither the fate of encecalin when incorporated by the beetles, nor possible reasons for the remarkable resistance of *T. geminata* towards this chromene have ever been investigated before. In the present study we have analyzed the tissue distribution and metabolism of incorporated encecalin in adults of *T. geminata.* Evidence provided from this study suggests that the tolerance of *T. geminata* towards encecalin is due to the rapid excretion of this chromene from the gut as well as to the insect metabolism of encecalin, which is mainly converted into the more polar encecalol (**4**) instead of the corresponding epoxide (**6,** Figure 1), as previously observed for susceptible insects (Randriaminahy et al., 1992; Kunze, 1995).

METHODS AND MATERIALS

Beetles and Plant Material. Eggs and adults of *Trirhabda geminata* and live plant samples of *Encelia farinosa* were collected at Box Spring Mountain, Riverside, California, U.S.A., in April and May 1994. The beetles were main-



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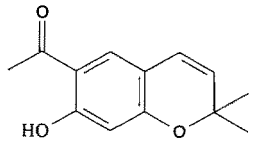

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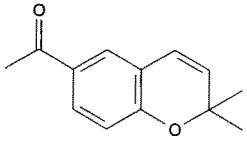

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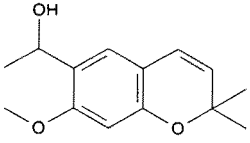

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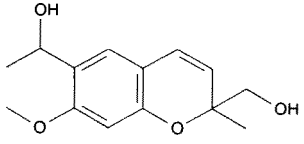

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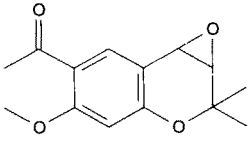

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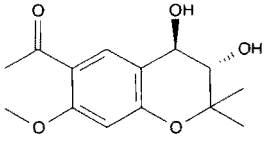

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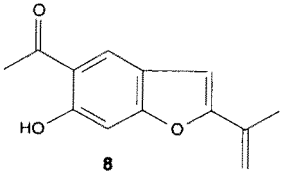

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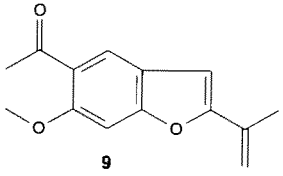

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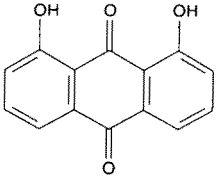

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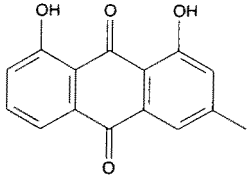

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FIG. 1. Structures of compounds **1–11.**

tained in the laboratory at room temperature (ca. 25°C) and allowed to feed freely on *Encelia farinosa* for 8 hr prior to their use in experiments.

Experimental Design. Groups of 20 beetles were starved for 0, 3, 6, 12, and 24 hr on wet tissue paper; the nonstarved control group consisted of 32 individuals. Hemolymph was collected from five beetles of each group and immediately dissolved in acetone. Frass and eggs were collected and freeze dried. Freeze-dried beetles and leaf samples were ground and extracted three times for 24 hr with acetone and shaking. The solvent was evaporated under vacuum, and the residue was dissolved in a known amount of methanol and filtered prior to HPLC and GC-MS analyses. Frass and eggs were ground and extracted once with acetone and filtered prior to HPLC and GC-MS analyses. The acetone solution of the hemolymph was filtered and analyzed by HPLC.

Sample Analysis. The HPLC system was from Knauer (Berlin, Germany) and equipped with a multi-wavelength detector (Knauer). Samples were injected on a Eurospher 100-C18 column (Knauer) (125 × 4 mm, 5-μm pore size) and separated using a linear binary gradient consisting of solvent A (H_2O, adjusted with orthophosphoric acid to pH 2) and solvent B (methanol) (from 100% A to 100% B in 45 min followed by an isocratic segment at 100% B for 15 min). Flow was at 1 ml/min, with detection at 254 nm. Encecalin, euparin, and methyleuparin were quantified using the external standard method. Encecalin and euparin were isolated from *Encelia* as described previously and were identified by their ^1H NMR and mass spectra (Proksch and Rodriguez, 1982).

The GC-MS system was a Carlo Erba Mega 5160 gas chromatograph equipped with a quartz column (30 m × 0.32 mm; DB-1, J&W Scientific, Folsom CA), which was directly coupled to a quadruple mass spectrometer (Finnigan MAT 4515). Conditions: injector 250°C, temperature program 150–300°C at 6°C/min, split ratio 1:20, carrier gas He at 0.5 bar.

RESULTS

HPLC analysis of leaves from *E. farinosa* infested with *T. geminata* revealed the presence of the chromenes encecalin (**1**) and encecalol (**4**) as major hydroxyacetophenone derivatives, followed by comparatively small amounts of the chromenes demethylencecalin (**2**) and demethoxyencecalin (**3**) and the benzofurans euparin (**8**) and methyleuparin (**9**) (Figure 1, Table 1). The sesquiterpene lactone, farinosin, previously reported from *E. farinosa* (Geissman and Mukherjee, 1968), was only detected as a trace constituent in the analyzed samples of *E. farinosa*. Compounds were identified based on their MS data and retention times as well as by comparison with authentic standards isolated earlier (Isman et al., 1987a; Proksch et al., 1987; Randriaminahy et al., 1992).

Beetles collected from the leaves (t = 0 hr, Table 1) showed the same

TABLE 1. DISTRIBUTION, CONCENTRATION, AND ABSOLUTE AMOUNTS OF CHROMENES AND BENZOFURANS IN *T. geminata* AND ITS HOST PLANT *E. farinosa*[a]

	Abundance of chromenes and benzofurans (%)					
		T. geminata			Feces	
Compound	Leaves of *E. farinosa*	0 hr	3 hr	Hemolymph (0 hr)	0 hr	3 hr
1	59	56	tr	ND	25	12
2	3	1	ND	ND	1	ND
3	6	1	ND	ND	2	1
4	21	39	ND	ND	61	76
5	ND	ND	ND	ND	10	10
8	7	3	ND	ND	1	1
9	4	ND	ND	ND	ND	ND
Conc. (mg/g dry w)	42.1	2.7 ± 1.5				
Absolute amounts (μg/individual)		21.7 ± 10.9				

[a]Numbers of compounds refer to Figure 1. ND = not detected; tr = trace.

pattern of chromenes as present in *E. farinosa*. From the benzofurans of *E. farinosa* only euparin (**8**) was detected in *T. geminata*, suggesting that the beetles had transformed methyleuparin (**9**) into euparin (**8**) by demethylation. Hemolymph obtained from the beetles, however, was free of any chromene or benzofuran derivatives (Table 1), indicating that the ingested hydroxyacetophenone derivatives were apparently not absorbed across the gut membranes but remained largely in the alimentary canal.

Fecal pellets produced by *T. geminata* while feeding on leaves of *E. farinosa* (t = 0 hr, Table 1) were found to contain encecalin (**1**), demethylencecalin (**2**), demethoxyencecalin (**3**), encecalol (**4**), and euparin (**8**), as well as an additional chromene metabolite that was identified as 2-hydroxymethylencecalol (**5**) based on its mass spectral data (Proksch et al., 1987).

The relative amounts of encecalol (**4**) compared to the parent compound encecalin (**1**) were significantly higher in beetles as well as in fecal pellets of *T. geminata* when compared to leaves of *E. farinosa* (Table 1). Whereas the relative proportions of encecalin (**1**) and encecalol (**4**) amounted to 59% and 21% in leaves, the ratio shifted to 56%:39% in the beetles (t = 0 hr) and even to 25%:61% in the feces (t = 0 hr), indicating that reduction of the acetyl substituent was the major step in the metabolic fate of encecalin (**1**) when ingested by *T. geminata*.

Beetles that had been starved for 3 hr were found to be almost completely free of extractable chromene derivatives, whereas the fecal pellets produced during starvation contained encecalol (**4**) as the major chromene (76% of all identified hydroxyacetophenone derivatives) (Table 1). Encecalin (**1**) and the 2-hydroxymethylderivative of encecalol (**5**) were present as minor constituents (Table 1). Compared to the chromenes present in fecal pellets collected at t = 0 hr (Table 1) the relative amount of encecalol (**4**) had further increased during starvation at the expense of the parent compound encecalin (**1**). Two antraquinones (**10** and **11**) detected in *T. germinata* are apparently biogenetic products of the beetles since no anthraquinone derivatives were found in *E. farinosa*.

DISCUSSION

Chrysomelid beetles *T. geminata* are oligophagous herbivores that are well adapted to feeding on their host plant *E. farinosa* (Wisdom, 1985; Paine et al., 1993), in spite of its noxious natural products such as encecalin (**1**), which is well known for its insecticidal activity (Isman and Proksch, 1985; Srivastava and Proksch, 1991). The resistance of *T. geminata* towards encecalin is apparently closely correlated to the fate of this chromene when ingested by the beetles. HPLC analysis of hemolymph samples from beetles feeding on *E. farinosa* indicated that encecalin (**1**) or other hydroxyacetophenones are not absorbed from the gut (Table 1) but are largely confined to the alimentary canal (Table 1). Furthermore, ingested chromenes are rapidly cleared from the gut via the feces. Beetles that had been starved for only 3 hr were almost completely devoid of any detectable chromene derivatives (Table 1). In comparison, larvae of the generalist herbivore *Spodoptera littoralis* (Noctuidae), which are susceptible to the detrimental effects of chromenes, were previously shown to absorb encecalin (**1**) from the gut when feeding on artificial diet spiked with sublethal amounts of encecalin (Randriaminahy, 1992). When transferred to an encecalin-free diet, larvae of *S. littoralis* took more than 24 hr to completely excrete previously ingested encecalin (Randriaminahy et al., 1992; Randriaminahy, 1992).

Further difference between *T. geminata* and larvae of *S. littoralis* were observed with regard to metabolism of ingested encecalin. Whereas encecalol (**4**) was identified as major metabolite of encecalin in *T. geminata* (Table 1) the 3,4-*trans*-diol (**7**) was the predominating metabolite of encecalin (**1**) detected in feces of *S. littoralis* (Randriaminahy et al., 1992; Kunze, 1995). Encecalol as well as the 3,4-diol (**7**) are more polar and significantly less toxic than their parent compound and can be regarded as detoxification products of encecalin (Isman et al., 1987b). However, the diol (**7**) formed by larvae of *S. littoralis* originates from the unstable and cytotoxic 3,4-epoxide (**6**). Metabolism of ingested encecalin by larval mixed function oxidases (MFOs) is, in fact, req-

uisite for the toxicity of encecalin to larvae of *S. littoralis* since simultaneous treatment of larvae with encecalin and the powerful MFO inhibitor tetcyclasis was shown to increase larval survival significantly when compared to larvae treated with encecalin alone (Kunze, 1995). Thus, in larvae of *S. littoralis* encecalin is apparently subject to suicide metabolism similar to that of pyrrolizidine alkaloids in mammalian liver cells (Röder, 1984), whereas the resistant *T. geminata* beetles metabolize encecalin primarily by reduction of the acetyl group and apparently not by epoxidation of the 3,4-double bond as judged from the recovered metabolites.

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ATTRACTIVENESS TO MEXICAN FRUIT FLIES OF COMBINATIONS OF ACETIC ACID WITH AMMONIUM/AMINO ATTRACTANTS WITH EMPHASIS ON EFFECTS OF HUNGER

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Abstract—Ammonium acetate was more attractive than other ammonium salts to Mexican fruit flies (*Anastrepha ludens*) in an orchard test. We hypothesized that acetic acid enhanced the attractiveness of ammonia in the orchard test and that acetic acid may similarly enhance attractiveness of AMPu, an attractant consisting of a mixture of ammonium bicarbonate or ammonium carbonate, methylamine HCl, and putrescine. In laboratory experiments, acetic acid was attractive to flies deprived of either yeast hydrolysate or both sugar and yeast hydrolysate but not to flies fed both sugar and yeast hydrolysate. AMPu/acetic acid combinations were more attractive than AMPu alone to flies deprived of both sugar and yeast hydrolysate but not to flies fed sugar, regardless of yeast hydrolysate deprivation status. Acetic acid is the first attractant found that has become more attractive with both sugar and protein deprivation in studies with *A. ludens*. It is also the first that has enhanced the attractiveness of another attractant type. In orchard tests, yellow sticky panels baited with either AMPu or 17 mg of acetic acid were at least six times more attractive than unbaited panels. However, panels baited with both acetic acid (17–68 mg) and AMPu were less attractive than AMPu alone. These results differed from the laboratory data in which combinations were never less attractive than AMPu alone.

Key Words—Attractants, Mexican fruit fly, Diptera, Tephritidae, *Anastrepha ludens*, ammonia, methylamine, putrescine, acetic acid.

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INTRODUCTION

Ammonia has been used as a trap bait for Tephritidae since at least the 1920s (Ripley and Hepburn, 1929; Jarvis, 1931), predating even protein baits (McPhail, 1939; Steiner, 1952). Numerous ammonium salts have been used as sources of ammonia: ammonium sulfate (Prokopy and Economopoulos, 1975); ammonium bicarbonate (Haniotakis and Vassiliou-Waite, 1987); ammonium phosphate (monobasic) (Stavrakis, 1970); ammonium phosphate (dibasic), ammonium carbonate, ammonium oleate, and ammonium chloride (Gothilf and Levin, 1989); and ammonium acetate [Hodson (1943), Reissig (1974) and Prokopy (1975) with *Rhagoletis pomonella* Walsh; Burditt et al. (1983) with *Anastrepha suspensa* (Loew); Hedstrom and Jimenez (1988) with *A. obliqua* Macquart and *A. striata* Schiner; Gothilf and Levin (1989) and Heath et al. (1995) with *Ceratitis capitata* Wiedemann]. The great number of investigations using ammonium acetate as a fruit fly attractant testifies to its superior attractiveness relative to other ammonium salts.

Results of the above studies suggest that acetic acid, the form in which acetate volatilizes from solution, is the key to the superior attractiveness of ammonium acetate. Acetic acid has been reported attractive to *C. capitata* (Keiser et al., 1976) and to *Anastrepha ludens* Loew (Robacker and Flath, 1995). As discussed above, ammonia has long been acknowledged as an attractant for fruit flies. Therefore, we hypothesized that attractiveness of ammonium acetate probably was due to combined attractiveness of ammonia and acetic acid.

Robacker and Warfield (1993) described a three-component attractant (AMPu) for *A. ludens* that also utilizes ammonia as an attractive principal. AMPu consists of a mixture of ammonium bicarbonate or ammonium carbonate, methylamine HCl, and putrescine. AMPu was developed following the approach used by Wakabayashi and Cunningham (1991) to develop an attractant for *Bactrocera cucurbitae* Coquillett. The overall attractiveness of AMPu has been shown to be the result of additive effects of the three components.

In agreement with the above results, other studies in our laboratory have shown that combinations of attractive components within systems [for example, within the pheromone system (Robacker, 1988)] were more attractive than individual components to *A. ludens*. Conversely, combinations of chemicals from different systems (for example, pheromone with fruit odor) were either no more attractive or less attractive than the more attractive of the two systems (Robacker and Garcia, 1990). If ammonium acetate actually is more attractive than other ammonium salts, then we were interested in learning if AMPu/acetic acid combinations would also be more attractive than AMPu alone.

In addition to studies of component additivity on overall attractiveness, our laboratory has actively studied the effects of physiological state on responses of fruit flies to attractants in *A. ludens*. Most relevant to the current problem, effects

of hunger on attraction of flies to bacterial odor have been studied (Robacker and Garcia, 1993; Robacker and Moreno, 1995). Because bacterial odor contains acetic acid (Robacker and Flath, 1995), we wondered if hunger of flies would affect their attraction to acetic acid in the same way as to bacterial odor. The functional-group dissimilarity of acetic acid compared with the ammonia/amino compounds that comprise most of the attractive volatiles of bacterial odor suggested that acetic acid and bacterial odor would elicit different hunger-mediated responses.

The purposes of the current research were twofold: to determine the effects of sugar and protein hunger on attractiveness of acetic acid and AMPu to *A. ludens*, and to investigate interactions of acetic acid and AMPu with respect to their attractiveness, including effects of hunger on the interactions. The research was conducted as a series of three experimental paradigms. The first was a field test to verify that ammonium acetate was in fact more attractive than other ammonium salts to *A. ludens*. Next, we conducted laboratory experiments to determine effects of sugar and protein hunger on attraction of flies to AMPu, acetic acid, and their combination. Finally, field tests of AMPu, acetic acid, and their combination were conducted.

METHODS AND MATERIALS

Insects. A. ludens used in most experiments were from a culture that originated from mangoes collected in Morelos, Mexico, in 1953. Flies used in the orchard test of ammonium salts were from a culture that originated from yellow chapote fruit, a native host of the fly, collected in Nuevo Leon, Mexico, in 1987. Both cultures had been maintained on a laboratory diet since establishment. Flies used in orchard tests were irradiated with 70–84.7 Gy ^{137}Cs one to two days before adult eclosion for release into the orchard, to comply with quarantine laws for releasing *A. ludens*. Flies used in laboratory bioassays were not irradiated. Mixed-sex groups of 150–200 flies were kept in 0.5-liter cardboard cartons with screen tops until used in tests. Laboratory conditions for holding flies were 20–25°C, 50–70% relative humidity, and photophase from 06:30 to 19:30 hr provided by fluorescent lights.

Chemicals. AMPu originally was developed as an aqueous solution of ammonium bicarbonate, methylamine HCl, and putrescine in a 10:10:1 ratio (Robacker and Warfield, 1993). More recently, ammonium carbonate was substituted for ammonium bicarbonate because of its greater solubility in water, keeping the molar ratios of ammonia, methylamine, and putrescine the same as in the original AMPu (Robacker, 1995). Ammonium bicarbonate, methylamine HCl, and putrescine were obtained from Sigma Chemical Co. (St. Louis, Missouri), ammonium carbonate from Aldrich Chemical Co., Inc. (Milwaukee,

Wisconsin), and glacial acetic acid from Mallinckrodt Specialty Chemicals Co. (Paris, Kentucky). All chemicals were at least 98% pure.

Orchard Tests of Ammonium Salts (Experiment 1). A mixed citrus orchard located near the laboratory in Weslaco, Texas, was used for all field experiments. The orchard contained several varieties of orange, lemon, grapefruit and tangerine trees of varying ages. A section of the orchard containing Rio Red grapefruit (*Citrus paradisi* MacFadyen) was used for this experiment. Six treatments were tested in a 6 × 6 Latin-square design. Treatments were: 1% ammonium acetate; 5% ammonium phosphate (monobasic); 2.5% ammonium lactate; 2.5% ammonium sulfate; 2.5% ammonium citrate (dibasic); and 10% NuLure (Miller Chemical and Fertilizer Corp., Hanover, Pennsylvania) with 3% borax, a standard protein-hydrolysate bait for fruit flies. These concentrations of ammonium salts were more attractive than others tested in preliminary laboratory tests.

Treatments were prepared in deionized water. All except NuLure contained 0.01% Triton X-100 (Rohm and Haas Co., Philadelphia, Pennsylvania) as a surfactant. Treatments were tested in plastic McPhail-type traps, 200 ml of each per trap. Traps were hung one to a tree, 1–2 m aboveground, on the northeast sides of trees. Traps were placed in alternate trees within rows, and in alternate rows within the orchard. Positions of treatments were randomized within rows and columns. Flies were released into the orchard when 3–4 days old during the evening of the day before a test. Flies were fed a 6% sucrose solution up until the time of release. Approximately 5000 flies were distributed equally among the 36 test trees. Traps were placed in the orchard during the morning and removed for fly counts and cleaning two days later. The experiment was not repeated.

Laboratory Bioassays (Experiments 2–4). Three experiments were conducted to assess effects of sugar and protein hunger on attractiveness of AMPu, acetic acid, and their combination using cage-top bioassays. Attractiveness of acetic acid to Mexican fruit flies fed sugar but not protein was established in earlier work (Robacker and Flath, 1995). Of four quantities tested, 10 μg of acetic acid was the most attractive. Therefore, this quantity was used in the current research. AMPu was also tested at quantities of chemicals that were most attractive in previous work (Robacker and Warfield, 1993): ammonium bicarbonate, 10 μg; methylamine HCl, 10 μg; and putrescine, 1 μg. Both acetic acid and AMPu were prepared in water. The pH of the AMPu solution was adjusted to 8.8 with NaOH, a pH that was highly attractive in previous work (Robacker and Warfield, 1993). Test quantities of both acetic acid and AMPu were administered to flies in 10 μl of the aqueous solutions.

In the first laboratory experiment (experiment 2), flies fed ad libitum from eclosion on standard adult-fly diet, a mixture of sucrose (sugar from local grocery store) and enzymatic yeast hydrolysate (U.S. Biochemical Corp., Cleveland, Ohio). Additional sugar cubes were provided so that flies could regulate

the amount of carbohydrate and protein they received. In experiment 3, flies fed ad libitum on sugar cubes only. Experiment 4 was conducted like experiment 3 except sugar cubes were removed 48 hr before bioassays. Flies in all three experiments had access to water at all times.

Bioassays were conducted by placing four filter paper triangles (3 cm/side), one containing acetic acid, one with AMPu, one with acetic acid and AMPu, and one with water (10 μl), near the corners on the top of an insect cage (30 cm/side, aluminum-screened). For the papers containing both acetic acid and AMPu, the 10-μl quantities of each were loaded as separate spots so that non-volatile acetate salts did not form on the papers. Five counts of flies were made, at 1-min intervals, at each of the four treatments in their initial positions on the four corners of the cage-top. Then new papers with new loadings of the same four treatments were placed on the cage-top but rotated by 90° into new positions for five more counts, and so on, until all four treatments occupied each of the four positions for five counts. Totals of the 20 counts at each treatment were calculated, and these totals were used in statistical analyses. The filter papers were raised 5 mm above the cage top using plastic rings to ensure that olfaction and not contact chemoreception was solely responsible for the response of the flies.

One carton of 180–200 flies was used in each bioassay cage. Flies were tested when 5–9 days old. Tests were conducted between 10:00 and 14:00 hr under a combination of fluorescent and natural light. Sixteen replications of experiment 2, 14 replications of experiment 3, and 12 replications of experiment 4 were conducted.

Orchard Tests of AMPu and Acetic Acid (Experiments 5 and 6). One row of Ruby Red grapefruit (*C. paradisi*) and one row of Dancy tangerine (*C. reticulata* Blanco) were chosen for tests. Two linear blocks of eight consecutive trees each were used in each row, for a total of four blocks in the orchard.

AMPu and acetic acid were formulated into agar (Bacto Agar, Difco Laboratories, Detroit, Michigan) in 1.9-ml polypropylene microcentrifuge tubes (A. Daigger & Company, Inc., Wheeling, Illinois). Both the AMPu/agar tubes and the acetic acid/agar tubes were prepared by mixing hot agar solution with aqueous AMPu or acetic acid solutions in the microcentrifuge tubes to a volume of 1.7 ml. Final agar concentration was 1% in both AMPu and acetic acid tubes. AMPu concentrations in agar tubes were 60 mg/ml ammonium carbonate, 100 mg/ml methylamine HCl, and 10 mg/ml putrescine. Final pH of the AMPu tubes was 8.5–8.8. Acetic acid tubes were prepared at 10 concentrations of acetic acid, five of which were tested in experiment 5 and five in experiment 6. Different concentrations of acetic acid were tested in an attempt to test a range that included concentrations that were too low to have any effect up to concentrations that were so high as to be repellent. Concentrations tested in experiment 5 were 2.5, 5.0, 10, 20, and 40 mg/ml. Concentrations tested in experiment 6 were

0.06, 0.12, 0.25, 0.50, and 1.0 mg/ml. Experiment 6 used lower acetic acid concentrations because results of experiment 5 indicated that concentrations may have been too high.

Eight treatments were tested in each block. Experiment 5 treatments consisted of unbaited, AMPu, and acetic acid at 10 mg/ml and five combinations of the single concentration of AMPu and one of the five higher concentrations of acetic acid (2.5–40 mg/ml). Experiment 6 treatments consisted of unbaited, AMPu, and acetic acid at 0.25 mg/ml and five combinations of AMPu with one of the five lower concentrations of acetic acid (0.06–1.0 mg/ml). AMPu tubes and acetic acid tubes were fastened with their caps open to the tops of yellow panel traps (13 × 18 cm) (Robacker, 1992). For traps containing combinations of AMPu and acetic acid tubes, the two tubes were placed on opposite sides of the trap. Traps were coated with Tangle-Trap (Tanglefoot Company, Grand Rapids, Michigan) and were hung one to a tree, north of center, at 1–2 m height.

Flies were released into the test orchard when 4–14 days old during the late afternoon of the day before a test. Flies were fed sucrose and water until the time of release. Approximately 2000 flies were distributed equally among the 32 test trees in the four blocks. Traps were placed in the orchard during the morning and removed for fly counts and cleaning on the following morning. Traps were reused after removing flies and applying Tangle-Trap as necessary. Positions of treatments within each block were randomized for the first replication of each experiment. Positions of treatments in consecutive replications were not randomized but were moved sequentially within each block. Ten replications of experiment 5 and eight replications of experiment 6 were conducted.

Statistical Analyses. The field test of ammonium salts (experiment 1) was analyzed by analysis of variance (ANOVA) for a Latin-square design. Count data were analyzed after transformation to natural logarithms. Treatment means were compared by Fisher's protected least significant difference (LSD) method. These analyses and those described below were conducted using SuperANOVA (Abacus Concepts, 1989).

Two-way ANOVA was conducted for the three laboratory experiments (experiments 2–4) to separate effects of replication (each bioassay) and test chemical or chemical combination. Data used in ANOVAs were total counts at each treatment or water control for each bioassay resulting in counts of 16, 14, and 12 for experiments 2, 3, and 4, respectively. Means separations were by Fisher's protected LSD.

Separate one-way ANOVAs were conducted for males, females, and males plus females for each of the two field experiments of AMPu/acetic acid (experiments 5 and 6). Counts from traps were converted to proportions, then transformed by arcsin of the square root before ANOVAs were conducted. For example, the proportion of males captured in a particular trap on a particular

day was calculated as the number of males in that trap divided by the number of males captured in all traps in the block containing that trap on that day. Proportions were used to eliminate the high variability in capture rates from day to day because tests were conducted over two seasons. Means separations were by Fisher's protected LSD.

Sex ratios of flies captured by the lures were compared for experiments 5 and 6. Totals of females and males captured on each trap date by each lure type were used to calculate the proportion of females captured by each lure type per day. Data were analyzed by one-way ANOVA without transformation.

RESULTS

Orchard Tests of Ammonium Salts (Experiment 1). Ammonium acetate captured significantly more flies than the other ammonium salts (Figure 1). Only NuLure was comparable in attractiveness. A total of 1077 flies were captured in this experiment.

Laboratory Bioassays (Experiments 2–4). Acetic acid was no more attractive than water to flies fed both sugar and yeast hydrolysate until the time of testing (experiment 2) (Figure 2). Acetic acid was significantly more attractive than water to flies fed sugar but not protein (experiment 3) and to flies that were hungry for both sugar and protein (experiment 4). AMPu was significantly more attractive than both water and acetic acid to flies of all hunger-status groups.

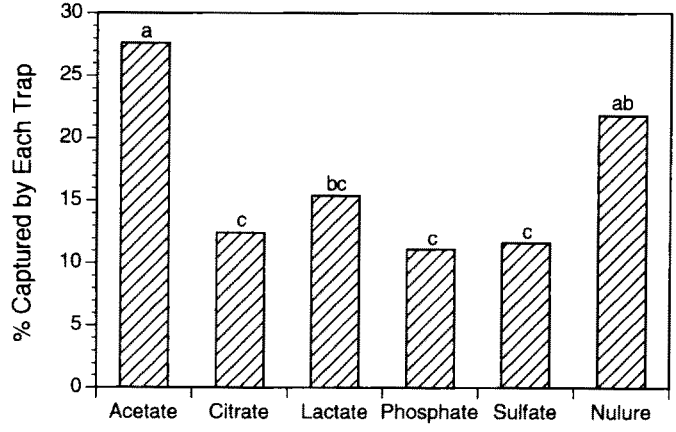

FIG. 1. Captures of *A. ludens* in McPhail-type traps containing solutions of various ammonium salts or NuLure (experiment 1), expressed as mean percentages of the total flies captured by the six treatments. Bars with the same letter are not significantly different by Fisher's protected LSD ($P < 0.05$).

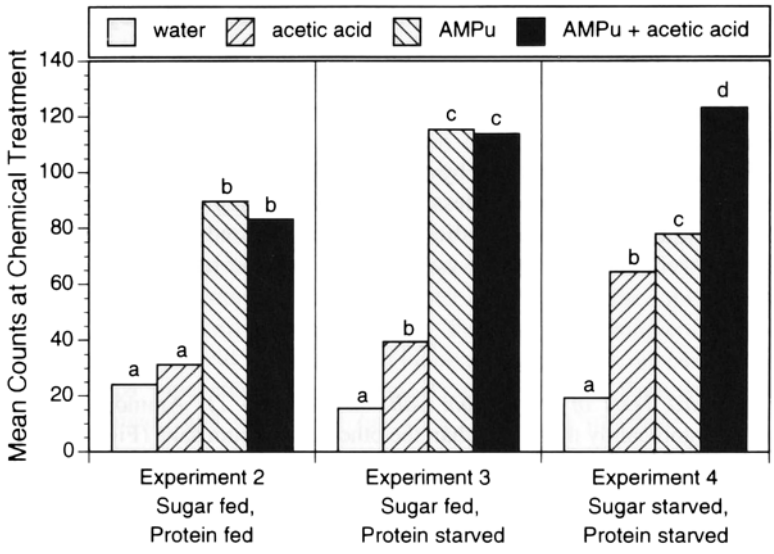

FIG. 2. Mean counts of *A. ludens* of three hunger states at filter papers containing water, acetic acid, AMPu, or an AMPu/acetic acid combination in cage-top bioassays. Within each experiment, bars with the same letter are not significantly different by Fisher's protected LSD ($P < 0.05$).

The combination of acetic acid and AMPu was more attractive than AMPu alone to flies hungry for both sugar and protein, but not to the other hunger-status groups of flies.

Orchard Tests of AMPu and Acetic Acid (Experiments 5 and 6). Experiment 5 showed that yellow panel traps baited with 17 mg (10 mg/ml × 1.7 ml) of acetic acid were considerably more attractive than unbaited yellow panel traps (Figure 3). AMPu-baited traps were not significantly more attractive than acetic acid-baited traps. Traps baited with combinations of acetic acid ranging in concentration from 2.5 to 40 mg/ml and AMPu were generally less attractive than traps baited with AMPu alone. The combinations were significantly less attractive than AMPu alone at the three highest concentrations of acetic acid. A total of 930 flies were captured in this experiment, summed over all treatments and replications.

In experiment 6, AMPu traps captured six times as many flies as unbaited traps, about the same as in experiment 5. The ANOVA was highly significant ($F = 16.2$; $df = 7,248$; $P < 0.0001$). Traps baited with 0.4 mg (0.25 mg/ml × 1.7 ml) of acetic acid were not significantly more attractive than unbaited

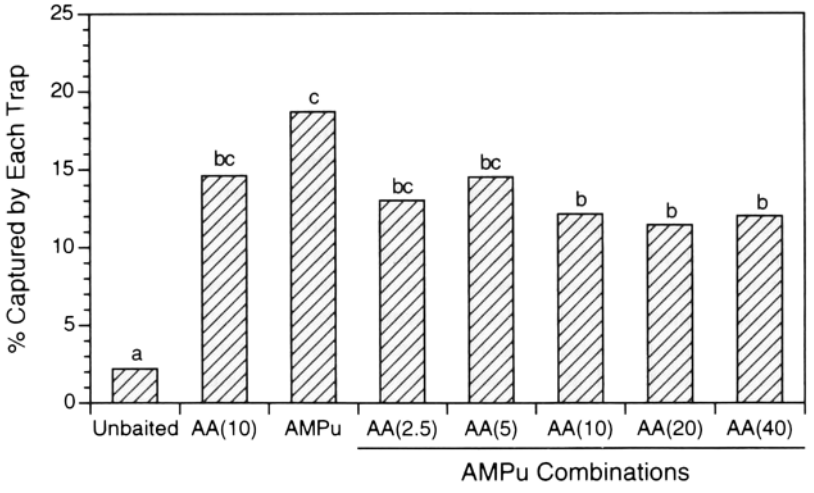

FIG. 3. Captures of *A. ludens* on sticky panel traps baited with nothing (unbaited), 17 mg of acetic acid [AA(10) = 10 mg/ml × 1.7 ml], AMPu, or combinations of AMPu with five amounts of acetic acid (experiment 5), expressed as mean percentages of the total flies captured by the eight treatments. Bars with the same letter are not significantly different by Fisher's protected LSD ($P < 0.05$).

traps by Fisher's protected LSD at the 5% level. Traps baited with combinations of acetic acid ranging in concentration from 0.06 to 1 mg/ml and AMPu also were not significantly different from traps baited with AMPu alone by LSD ($P < 0.05$). A total of 1640 flies were captured in this experiment, summed over all treatments and replications.

Most of the trap/lure combinations captured more females than males. The percentage of males and females in released flies was not recorded so it was not possible to determine if the various lures captured females or males in significantly different proportions than were present in the orchard. However, Robacker and Warfield (1993) showed that AMPu was approximately equally attractive to males and females.

We did compare the attractiveness to males vs females of the various lures relative to each other. In experiment 5, percentages of females captured by the various lures were unbaited, 52.4%; acetic acid, 49.6%; AMPu, 61.4%; and all AMPu/acetic acid combinations, 65.4%. In experiment 6, percentages of females captured by the lures were unbaited, 54.5%; acetic acid, 60.7%; AMPu, 57.6%; and all AMPu/acetic acid combinations, 59.5%. ANOVAs of proportions of females captured by traps per test day showed no significant differences among the lures.

DISCUSSION

Ammonium Acetate. The great attractiveness of ammonium acetate compared to the other ammonium salts suggests that acetic acid plays a role in attractiveness of ammonium acetate. The results of the experiments in which acetic acid was combined with AMPu proved that acetic acid affected overall attractiveness of these combinations. Based on the experiments with AMPu, it is more prudent to argue that acetic acid affected attractiveness of ammonium acetate than to argue otherwise. We cannot argue with certainty that acetic acid was the primary reason that ammonium acetate was more attractive than the other ammonium salts tested in this work because no attempt was made to equalize emissions of ammonia from the various preparations. However, each compound was tested at a concentration that ensured optimum performance based on preliminary testing.

Effects of Hunger on Attractiveness. The attractiveness of AMPu, acetic acid, and AMPu/acetic acid combinations was shown to be affected by the hunger status of flies. AMPu was attractive to flies of all hunger states. Although results of the experiments could not be compared statistically with each other, it appeared that AMPu was most attractive to flies hungry for protein (experiment 3) and least attractive to flies hungry for sugar (experiment 4). Previously, Robacker and Garcia (1993) demonstrated that bacterial odor was not attractive to *A. ludens* that were hungry for sugar, and Robacker and Moreno (1995) demonstrated that bacterial odor was most attractive to flies hungry for protein. The similar result found here for AMPu is not surprising due to the similarity of attractive principals in AMPu and bacterial odor (Robacker et al., 1993; Robacker and Flath, 1995).

Data for acetic acid revealed a very different relationship between hunger and attractiveness compared with AMPu. Acetic acid became more attractive as hunger for either sugar or protein increased. This relationship is also different from that of CEH, a three-component attractant (1,8-cineole, ethyl hexanoate, and hexanol) developed from odor of fermented host fruit (Robacker et al., 1990b). Like acetic acid, attractiveness of CEH increased with sugar deprivation of flies (Robacker, 1991). However, protein deprivation had little effect on attractiveness of CEH. Acetic acid is the first chemical studied that became more attractive to *A. ludens* deprived of either sugar or protein. With these data as a base, acetic acid does not fit into either the sugar-hunger or protein-hunger attractant systems described by Robacker (1991).

The greater attractiveness of the AMPu/acetic acid combination relative to either attractant alone when flies were starved for both sugar and protein is also unique among attractants studied to date in *A. ludens.* It is the first time that a combination of two attractants containing such different chemical moieties, and which operate somewhat differently with respect to effects of hunger, was more

attractive than one or the other of the two attractants alone. However, this result only occurred when flies were hungry for sugar and may occur in the field only at times when natural sources of carbohydrate are scarce.

Orchard Tests of AMPu/Acetic Acid Combinations. Combinations of AMPu with acetic acid were either less attractive or no more attractive than AMPu alone in field tests. This result cannot be attributed to acetic acid acting as a repellent. Acetic acid was attractive when tested alone, as discussed above. The field data resemble the laboratory data in that AMPu was the most attractive lure, acetic acid generally was more attractive than blanks, and the AMPu/acetic acid combinations were generally not more attractive than AMPu alone. In other ways, however, these results resemble neither laboratory experiments 2 and 3, in which acetic acid was much less attractive than AMPu and AMPu/acetic acid combinations, nor laboratory experiment 4, in which the AMPu/acetic acid combination was significantly more attractive than AMPu. The reason for these differences between the laboratory and field tests is unknown. Previous research indicated that irradiation attenuated attraction of *A. ludens* both to odor of fermented host fruit (Robacker et al., 1990a) and to bacterial odor (Robacker and Garcia, 1993) by similar amounts. What effects irradiation of flies used in field tests in this work may have had on relative attraction to acetic acid, AMPu, and their combinations cannot be predicted.

Diminution of attractiveness when attractants were combined has been reported before in *A. ludens.* Robacker and Garcia (1990) reported that the combination of attractive odor of fermented chapote fruit and male-produced pheromone was much less attractive to sexually mature virgin females than was pheromone alone. That work also showed that the combination was less attractive than chapote odor alone to sexually immature females. Robacker (1991) showed that a combination of CEH and bacterial odor was less attractive than CEH alone to 2- to 3-day-old flies that had not been fed as adults. In *B. oleae* (Gmelin), combinations of pheromone and proteinaceous feeding attractants were also found to be less attractive than the proteinaceous attractants alone (Haniotakis and Skyrianos, 1981; Zervas, 1989) or pheromone alone (Haniotakis and Vassiliou-Waite, 1987).

Zervas (1989) overcame the repellency problem of the pheromone/yeast hydrolysate combination by moving the pheromone dispenser 0.5–1 m away from the trap. This trapping system resulted in an increase in the capture of *B. oleae* compared to either bait alone. In the current study with *A. ludens,* putting AMPu vials and acetic acid vials on opposite sides of the panel traps did not result in an increase in the number of flies captured compared with AMPu alone. No attempt was made to duplicate Zervas' (1989) results by moving the AMPu and acetic acid lures 0.5–1 m apart.

As discussed above, Zervas (1989) successfully increased captures of *B. oleae* by combining two different types of attractants. In addition, Landolt et

al. (1992) demonstrated that a combination of pheromone from *Toxotrypana curvicauda* Gerstaecker with host-fruit odor was more attractive to females than either pheromone or host-fruit odor alone. Even more relevant to the current work, MacCollom et al. (1992, 1994) found that a combination of bacterial cells with apple volatiles was more attractive than either alone to *R. pomonella* in an apple orchard. These results indicate that it should be possible to develop more powerful attractants for at least some species of Tephritidae by combining different types of attractants. However, no evidence has been found to suggest that combining various types of attractants for the Mexican fruit fly, including AMPu with acetic acid, as shown in this paper, will enhance capture of the flies beyond captures by AMPu or other nitrogenous types of attractants alone.

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IDENTIFICATION AND BIOASSAY OF KAIROMONES FOR *Helicoverpa zea*

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Abstract—Hexane extracts of leaves of 307 accessions from 73 host plant species of *Helicoverpa zea* were analyzed by gas chromatography (GC) and used for *H. zea* oviposition and neonate larvae orientation bioassays. The gas chromatographic (GC) retention times of compounds statistically associated with behavioral activity were identified by correlation of GC peak area with oviposition and larval orientation preferences. Although taxonomically diverse in their origin, compounds for study were purified from extracts of species of the genus *Lycopersicon*, due to their relative abundance. The structures of eight long-chain alkanes associated with oviposition preference were assigned by mass spectrometry, and the structures of five similarly associated organic acids and a terpenoid alkene were identified by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. The structures of a number of other phytochemicals from the plant leaves were identified for comparative purposes, including a previously unknown terpene, 7-epizingiberene. Bioassays were performed on the isolated acids and on the alkane wax fractions of several *Lycopersicon* species, and significant differences were found in oviposition stimulation for both classes of compounds. Of the hundreds of compounds found in the extracts, none were observed to act as oviposition deterrents. The results of these bioassays may be useful in explaining the broad host range of *H. zea*, as well as the process and evolution of host plant selection for oviposition.

Key Words—Oviposition, kairomone, *Helicoverpa zea*, corn earworm, *Lycopersicon*, *Nicotiana*, host-plant selection, insect chemoreception, Lepidoptera, Noctuidae.

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INTRODUCTION

The larvae of *Helicoverpa zea* are a major agricultural pest in the United States and Central and South America, with damage and control costs for *H. zea* and *H. virescens*, a related pest, estimated to be one billion dollars each year. Their host plants include a variety of economically important crops such as corn, tomatoes, cotton, tobacco, and soybeans (Johnson et al., 1986). Traditionally, *H. zea* has been controlled primarily through the repeated application of pesticides, but ecological and economic costs of pesticides and the possibility of insecticide-resistant strains of *H. zea* have led to an increased interest in other pest management strategies. Some of these strategies depend on research in *H. zea* semiochemicals, with the eventual goal of modifying growers' cultural practices or genetically manipulating host species to produce naturally resistant plants.

One focus of research has been insect pheromones. Pheromones have been used with varying degrees of success to bait field traps to monitor *H. zea* pest populations (Leonard et al., 1989; Raulston et al., 1990). A second focus involves research in plant/insect semiochemicals (kairomones and allomones), as well as plant-produced mimics of insect hormones. Because the larvae are the agricultural pests, one reasonable approach to control of *H. zea* involves identifying oviposition stimulants. This could lead to a number of insect control strategies, including improved monitoring of female *H. zea* populations in the field, possible disruption of egg-laying behavior, and cultivation of host species that do not produce the stimulants so that the moths would no longer recognize crop plants as hosts for oviposition.

Although many stimuli are involved in lepidopteran oviposition behavior, chemical stimulation is considered the major factor (Städler, 1986), with visual and mechanical stimuli also playing roles. In their study of the attraction of *H. subflexa* to plant extracts, Tingle et al. (1989) noted that the flight of mated females (but not males or virgin females) was particularly directed toward extracts of leaves of *Physalis* and that the total positive response to plume trails of leaf extract, including anemotaxis, contact, and landing, was greater than 80%. Thus, identification and manipulation of the leaf chemical components may allow for potential manipulation of oviposition behavior.

A number of natural and synthetic oviposition stimulants for *H. zea* and related pests have been identified. Jones et al. (1970) discovered that the triester triacetyl glycerol, a component of some pen inks, stimulated significant levels of oviposition activity. Jones et al. (1973) tested 141 compounds structurally related to triacetyl glycerol, including esters and halogenated esters, and found that 26 of them stimulated oviposition in lab tests, but none showed any activity in field tests. Wiseman (1989) found that chemicals from corn silk stimulated oviposition of *H. zea*. Mitchell et al. (1990) tested methanol and methylene chloride leaf washes from ground cherry (*Physalis angulata*), and the *H. zea*

host plants tobacco (NC 2326), *Desmodium tortuosum*, and cotton (*Gossypium hirsutum*) for oviposition activity with *H. zea*, *H. subflexa*, and *H. virescens*. They found that while *H. subflexa* was stimulated by groundcherry and *H. virescens* by tobacco, *H. zea* was not influenced by any of the extracts.

Previous work in our labs has centered on β-bergamotenoic acid, and the structurally similar α-bergamotenoic and α-santalenoic acids, compounds exuded from trichomes on the leaf surface of *Lycopersicon hirsutum* (Coates et al., 1988; Juvik et al., 1988). β-Bergamotenoic acid in particular was found to stimulate oviposition activity in *H. zea*. A number of bicyclic and acyclic analogs of β-bergamotenoic acid were synthesized and tested for oviposition activity. The acid functionality was essential for oviposition stimulation, since none of the alkene, ester, or acetate analogs of β-bergamotenoic acid had activity (Douglass et al., 1993).

Part of the problem associated with identifying kairomones for *H. zea* is the diversity of its host range and of the chemical constituents present among many host-plant species. A single accession of a host species may have more than 70 different compounds in its leaf surface extracts (Juvik, unpublished data). The goal of the present work was to implicate, by statistical correlation of gas chromatographic and oviposition bioassay data, larval attractants and oviposition stimulants for *H. zea* from a broad range of its host plants. Structural determinations and *H. zea* oviposition bioassays were then performed on putative kairomonal compounds isolated from the leaves of the plant family Solanaceae, primarily from species of *Nicotiana* and *Lycopersicon*.

METHODS AND MATERIALS

Plant Material and Extracts

Seeds of wild and cultivated *Lycopersicon* species were obtained from Dr. C. M. Rick, Tomato Genetics Stock Center, Department of Vegetable Crops, University of California, Davis, and from Dr. J. McFerson, Germplasm Resources Unit, United States Department of Agriculture (USDA), Geneva, New York; seeds of wild and cultivated *Capsicum* species were obtained from Dr. P. W. Bosland, Department of Agronomy and Horticulture, New Mexico State University, Las Cruces, New Mexico; seeds of wild *Gossypium* species were obtained from Dr. A. E. Percival, Cotton and Grain Crops Research Unit, USDA, College Station, Texas; seeds of tobacco and *Nicotiana* species were obtained from Dr. D. M. Jackson, Tobacco Research Laboratory, USDA, Oxford, North Carolina. All other seeds were purchased locally.

During the summers of 1988 and 1989, accessions from 73 species (Table 1) of plants known to host *H. zea* (Kogan, 1986) were grown in the greenhouses at the University of Illinois, Urbana-Champaign, and then transplanted into the

TABLE 1. HOST PLANTS OF *Helicoverpa zea* GROWN IN 1988 OR 1989 AND OVIPOSITION PREFERENCE VALUES OF LEAF HEXANE EXTRACTS FROM EACH ACCESSION

Family	Genus	Species	Number of accessions	Oviposition value (range)[a]
Solanaceae	*Lycopersicon*	*esculentum*	21	1.21–9.0
		esculentum var. *cerasiforme*	7	1.11–3.1
		pimpinellifolium	25	1.11–3.2
		cheesmanii	4	1.37–2.0
		parviflorum	6	1.53–2.4
		pennellii	13	1.69–5.5
		hirsutum f. *glabratum*	13	0.98–7.6
		hirsutum	44	1.48–9.4
		chmielewskii	7	1.38–6.5
		chilense	5	1.58–4.6
		peruvianum	16	1.40–5.7
	Solanum	*tuberosum*	2	1.29–2.0
		melongena var. *esculentum*	1	2.09
		pycanthum	1	1.87
		nigrum	1	1.20
		carolinese	1	1.57
	Physalis	*pruinosa*	1	1.85
		ixocarpa	1	2.45
	Petunia	*hybrida*	1	1.88
	Capsicum	*annuum*	15	1.04–7.1
		baccatum var. *baccatum*	2	1.30–2.1
		baccatum var. *microcarpum*	2	1.50–1.8
		baccatum var. *pendulum*	2	1.37–2.7
		chaloense	1	1.39
		chinense	1	1.40
	Nicotiana	*sylvestris*	1	1.31
		alata	1	1.91
		petunioides	1	1.73
		excelsior	1	1.35
		repanda	1	1.06
		knightiana	1	1.64
		glauca	1	2.26
		glutinosa	1	2.60
		rustica var. *brasilia*	1	1.92
		nudicaulis	1	1.43
		debneyi	1	2.16
		undulata	1	1.51
		trigonophylla	1	1.69
		tabacum	22	2.69–4.9
Leguminoseae	*Pisum*	*sativum*	3	1.28–2.6
	Lens	*esculenta*	1	2.74
	Phaseolus	*lunatus*	1	2.49
		vulgaris	3	1.45–3.1

TABLE 1. Continued.

Family	Genus	Species	Number of accessions	Oviposition value (range)[a]
	Arachis	*hypogea*	1	2.02
	Glycine	*max*	1	3.83
	Trifolium	*repens*	1	1.72
	Medicago	*sativa*	1	2.15
Cucurbitaceae	*Curcubita*	*pepo*	1	3.40
		maxima	3	1.63–2.73
		pepo var. *ovifera*	1	2.49
	Cucumis	*melo*	1	2.37
	Citrullus	*vulgaris*	1	1.63
	Cucumis	*sativus*	1	2.65
	Luffa	*cylindrica*	1	2.05
Cruciferae	*Brassica*	*oleracea* var. *botrytis*	2	1.99–3.66
		napus	2	2.53–4.39
		caulorapa	1	3.37
		oleracea var. *capitata*	1	2.36
		oleracea var. *gemmifera*	1	2.48
		chinensis	2	2.23–2.99
		juncea var. *crispifolia*	1	2.52
		oleracea var. *acephala*	1	2.08
Malvaceae	*Gossypium*	*hirsutum*	10	0.89–2.08
	Hibiscus	*hibiscus*	1	0.95
	Hibiscus	*esculentus*	1	1.10
	Abutilon	*theophrasti*	1	0.98
Compositae	*Taraxacum*	*officinale*	1	3.07
	Helianthus	*annuus*	1	2.29
Cannaceae	*Canna*	*generalis*	1	1.47
Amaranthaceae	*Amaranthus*	*retroflexus* (seed)	1	1.63
		retroflexus (foliage)	1	3.10
		tricolor var. *splendens*	1	1.98
Portulacaceae	*Portulaca*	*grandiflora*	1	1.89
Geraniaceae	*Pelargonium*	*hortorum*	1	5.33

[a]Oviposition values are the number of *H. zea* eggs on a disk treated with the hexane wash of the accession listed, divided by the mean number of eggs on two adjacent untreated disks.

field plots at the Agricultural Experiment Station South Farm. Leaves from each of the accessions were harvested two to three months later and transported to the laboratory. Leaf tissue (100 g) was placed in a canning jar with 400 ml of hexane and gently shaken for 30 min at room temperature. The extract was filtered through 24-cm Whatman No. 4 qualitative filter paper, and the extraction procedure was repeated using 100 ml of hexane. The extracts were combined

and concentrated to the equivalent of 0.5 g of leaf tissue per milliliter of hexane and stored in a −20°C freezer for subsequent analysis.

Accession Oviposition and Larval Orientation Bioassays

Gravid female moths of *H. zea* were obtained from our laboratory colony. Arenas for oviposition studies were used as described by Juvik et al. (1988). Hexane extracts for the various accessions were tested by placing 0.5 ml of hexane extract from each accession on 4.25-cm Whatman No. 1 filter paper disks and then allowing the hexane to evaporate. A control disk treated only with hexane, eight disks individually treated with extracts from eight accessions, and nine untreated disks were placed into arenas with 15 gravid female moths. Each treated and the control disk had two untreated disks adjacent to it. One of the nine treatments in each arena was the leaf extract from *Lycopersicon hirsutum* accession, LA 1777, which had been previously shown to stimulate *H. zea* oviposition (Juvik et al., 1988). Since it was known that similar concentrations of LA 1777 extract were highly attractive to the moths, it was used as a positive control, so that moth activity could be standardized over arenas and over days. In order to improve moth viability, a Petri dish containing sterile cotton infused with a 10% (v/v) honey in water solution was placed in the center of the bottom of each arena. The moths were allowed to oviposit for three days, with egg counts taken after each 24-hr period. Moth oviposition preference values were calculated by recording the number of eggs oviposited on the treated filter paper disks divided by the mean number of eggs on the two adjacent untreated disks. There were three replicates of each arena, with the positions of the treated disks for each arena randomized with respect to the other treated disks within that arena.

Larval bioassays were conducted by removing the eggs (including those on the filter paper disks) from the sides of the arenas used to determine moth oviposition preference values. These eggs were often damaged, and so were not used for the subsequent bioassay; however, enough eggs remained on the lid of the arena to provide an adequate hatch of larvae for the assays. There were approximately 5000 larvae per assay. Each of the treated disks was then resaturated with another 0.25 ml of extract of the same accession, allowed to dry, and returned to the arena. The eggs were allowed to hatch over a three-day period, and the number of the neonate larvae on each disk was counted at the end of each 24-hr period, at which point the larvae would be returned to the bottom of the arena by inducing anesthesia with a stream of CO_2. Larval orientation preference was calculated by dividing the number of larvae on each treated disk by the mean number of larvae on the two adjacent untreated disks. Larval bioassays were conducted only on the 226 accessions grown in 1988.

Statistics

The 1988 and 1989 GC data were tabulated by entering the retention times (corrected for drift by comparison with internal standard retention times) and peak area values for all peaks with areas more than or equal to a concentration of 0.005 mg 2-dodecanone/ml. Peak area values were standardized by dividing the actual integrated area by the integrated area of the 2-dodecanone internal standard, added just before GC analysis. Because many of the chemicals were present in a number of the extracts, the data entry procedure generated a matrix with retention times along one axis (as chemical identifiers), plant accession number along the second axis, and standardized peak areas for the individual chemicals as elements. For the 226 plant accessions in 1988, there were 310 chemicals identified by different GC retention times, with no one accession having more than 80 chemicals. For the 97 plant accessions in 1989, there were 171 chemicals recorded.

The oviposition bioassay data were entered as oviposition preference values, as calculated above. These values were standardized by dividing the oviposition preference value for a given accession by the oviposition preference value for LA 1777 for that arena minus the oviposition preference value for the hexane control for that arena, as shown in the following equation:

$$\text{Oviposition preference} = \frac{\text{Accession value}}{\text{LA 1777 value}} - \text{Control value}$$

Values for each of the three replicates were entered, for each of the three days of observation, for each accession. Larval bioassay data were entered as larval orientation preferences as defined above. They were standardized by dividing the larval orientation values for each accession by the larval orientation value for the hexane control disks. The number of larvae on LA 1777 was not used as an additional high standardization technique because we had no prior evidence that LA 1777 was attractive to *H. zea* larvae. As with the oviposition data, values for each of the three replicates were recorded for each of the three days of observation, for each accession.

Two-way analyses of variance (SPSS-X, 1990) of accession extracts, days, and their interaction were used to determine whether the replicates or the day of observation had a significant effect on standardized larval orientations or moth oviposition preferences. Because there were no significant main effects of replicates, day of oviposition, or their interaction, means were taken of the standardized values of oviposition and larva preference over the three days and three replicates.

For each year, correlations (SPSS-X, 1990) between oviposition preference values for each accession and standardized peak area of each chemical (as identified by GC retention time) in each accession were run to examine the variation

in oviposition preference values with the variation in standardized peak area. Only accessions with detectable amounts of a particular component were included in the regression for that component. For the 1988 standardized oviposition values, GC data from 16 chemicals that either individually accounted for a high proportion of total variability in the linear model ($R^2 > 0.40$) or had correlation coefficients whose P values were less than 0.05 were put into a subset. For the 1989 oviposition, 18 compounds fulfilled the above requirements, and their GC data were put into a subset. No negative correlations between compound concentrations and oviposition preference values were observed in these experiments.

Similarly, correlations (SPSS-X, 1990) between the larval preference values and the standardized peak area of each chemical in each accession were run to examine the variation in larval preference values with the variation in standardized peak area. For the larval orientation values, GC data from 17 chemicals that either accounted for a high amount of total variability in the linear model ($R^2 > 0.40$) or had a significant ($P < 0.05$) correlation with larval orientation were put into a subset.

Forward selection multiple regression (SPSS-X, 1990) was run on the data subsets to determine, for each year of oviposition and larvae preferences, which of the chemicals in each subset accounted for most of the total variability in activity. For 1988 standardized oviposition preference values, seven chemicals significantly increased the R^2 value of the regression equation ($P < 0.03$); for the larval standardized preference values, six chemicals significantly increased the R^2 value of the regression equation ($P < 0.002$); for 1989 standardized oviposition values, five chemicals significantly increased the R^2 value for the regression equation and were chosen for further analysis. In each of these three cases, the additional chemicals in the subsets had significantly greater P values, and so were not chosen for further investigation. GC-MS as described below was performed on the 18 putative kairomonal compounds (see Table 2 below) to determine their molecular weights and empirical formulas.

Chemical Analyses

Gas chromatography (GC) was performed on a Hewlett-Packard model 5790 gas chromatograph with a HP 7671A autosampler, a Hewlett-Packard Ultra Performance 12.5-m cross-linked methyl silicone capillary column (0.2 mm ID, with a 0.33-μ 100% dimethylpolysiloxane liquid phase) and a flame ionization detector. The carrier gas was helium; the split ratio was 100:1. The oven temperature was held at 90°C for 1 min, then raised by 10°C/min to 170°C, then 15°C/min to 320°C, and held at 320°C for 5 min. The injector was set at 325°C and the detector was maintained at 327°C. Internal standards (0.6 mg/

ml) of 2-dodecanone (Pfaltz and Bauer) and squalene (Aldrich) were used to standardize retention times by adjustment of retention times with internal standard retention time drift and peak integration values by comparison with internal standard GC peak integration. 2-Dodecanone was chosen as an internal standard because it elutes early in the GC run, separately from any naturally occurring compounds and is analogous to the 2-tridecanone and 2-undecanone found in *L. hirsutum* f. *glabratum* (Weston, 1989); squalene was chosen as an additional standard because it elutes much later in the run, separately from any of the naturally occurring compounds and so could be used for the standardization of compounds eluting at the end of the runs. Since a new column was used on the 1989 extracts, the chromatographic data from the two years were kept separate to avoid any possible misclassification of compound retention times.

Gas chromatography–mass spectrometry (GC-MS) was done in the Mass Spectrometry Laboratory of the School of Chemical Sciences at the University of Illinois. A Hewlett-Packard model 5890 GC was used, with the same column as described earlier. The runs were done as splitless injections, with helium as the carrier gas, under various oven conditions. A typical splitless run would be held at 35°C for 30 sec, then raised at 50°C/min to 90°C, then 10°C/min to 170°C, then 15°C/min to 320°C. The mass spectrometer used was a Fisons VG Analytical 70-VSE, with combined chemical ionization–electron impact ionization (CI-EI) capability, and a VG/OPUS data system. When samples were run in the ACE mode (alternating CI-EI mode, with 0.3 sec between scans), the ionization gas used was methane. For EI scans, the electron energy was 70 eV. High-resolution GC-MS was also performed on the same Fisons VG 70-VSE spectrometer. Electron ionization MS was used to observe fragmentation patterns, chemical ionization MS was used to confirm the molecular ions, and high-resolution MS was used to determine empirical formulas for the biologically active compounds. Fragmentation patterns of mass spectra were interpreted as discussed below.

Proton and carbon-13 nuclear magnetic resonance spectroscopy (^1H and ^{13}C NMR) were performed on a Varian XL200 NMR (200 MHz for ^1H) or on a Varian Unity 400 NMR (400 MHz for ^1H), as indicated. NMR chemical shifts were referenced to that for the deuterated solvent ($CDCl_3$ unless otherwise specified).

Flash chromatography was performed as described by Still (1978) using Merck 0.04 to 0.063-mm silica gel or Merck silica gel impregnated with 10% or 15% (w/w) silver nitrate. Thin-layer chromatographic (TLC) analysis was performed with glass plates precoated with 0.25 mm of silica gel F-254 with fluorescent indicator, manufactured by Merck. TLC plates for argentation chromatography were obtained from Alltech Associates, Inc. Plates were 20 × 20 cm, 250 μm thick, and coated with either 10% or 15% (w/w) silver nitrate–

silica gel. Plates were visualized by spraying with 5% phosphomolybdic acid in 95% ethanol and heating.

Reactions were carried out under a nitrogen atmosphere unless specified otherwise. All reagents and solvents were reagent grade and used without further purification unless specified otherwise. Hexanes were distilled prior to use. Ethereal diazomethane was produced from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald) in ether with potassium hydroxide in ethanol following the procedure described by Black (1983).

Identification of Alkane Waxes

The structures of the alkane waxes were determined by gas chromatography in conjunction with mass spectrometry (GC-MS) and by the use of standardized retention times, or Kovats indices (Kovats, 1965). The use of these indices, in conjunction with the carbon number as judged by GC-MS, was used to determine whether the particular alkane was branched or unbranched, and the extent of branching present. A similar technique relying on Kovats's work was used to show that the alkanes were members of homologous series. Because members of homologous series will elute from an isothermal GC run so that the logarithms of their retention times are proportional to the numbers of carbons in the molecules, a plot of the logarithm of isothermal retention time versus the carbon number will result in straight lines (Hutchins and Martin, 1968).

Interpretation of mass spectral fragmentation patterns according to rules described by Pomonis et al. (1980) and Nelson et al. (1980, 1984) was used to determine the number and position of methyl branches and whether a chromatographic peak represented a single component or a mixture of coeluting alkanes. Authentic samples of the *n*-alkanes pentacosane, hexacosane, heptacosane, octacosane, dotriacontane, and tritriacontane were obtained from Sigma Chemical Company, (St. Louis, Missouri) and were used to corroborate GC retention times, mass spectral fragmentation patterns, and the line generated from plotting the logarithms of the isothermal GC retention time versus carbon number for the natural *n*-alkanes. The Kovats indices of the authentic alkanes were the same as those for the natural alkanes, and they agreed with those in the literature (Pomonis and Hakk, 1984). Literature values for the Kovats indices of the 2- and 3-methyl alkanes showed these alkanes to be homologous with 2- and 3-methyl alkanes for which Kovats indices were available in the literature (Pomonis et al., 1980, 1989). GC coinjection of the authentic *n*-alkanes also helped corroborate the identification of the methyl branched alkanes, since it is known that the order of elution for alkane structural isomers is 2-methyl-, 3-methyl-, *n*-alkanes (Nelson, 1977). In all cases, comparison with the authentic samples confirmed the structural assignments given.

Chemical Identification

The structures of all the compounds used in these experiments except the fatty acids (7, 10, 13-hexadecatrienoic, palmitic, and linolenic acids) and the long-chain alkanes are shown in Figure 1.

Chemicals implicated as biologically active include: 2-methyldotriacontane

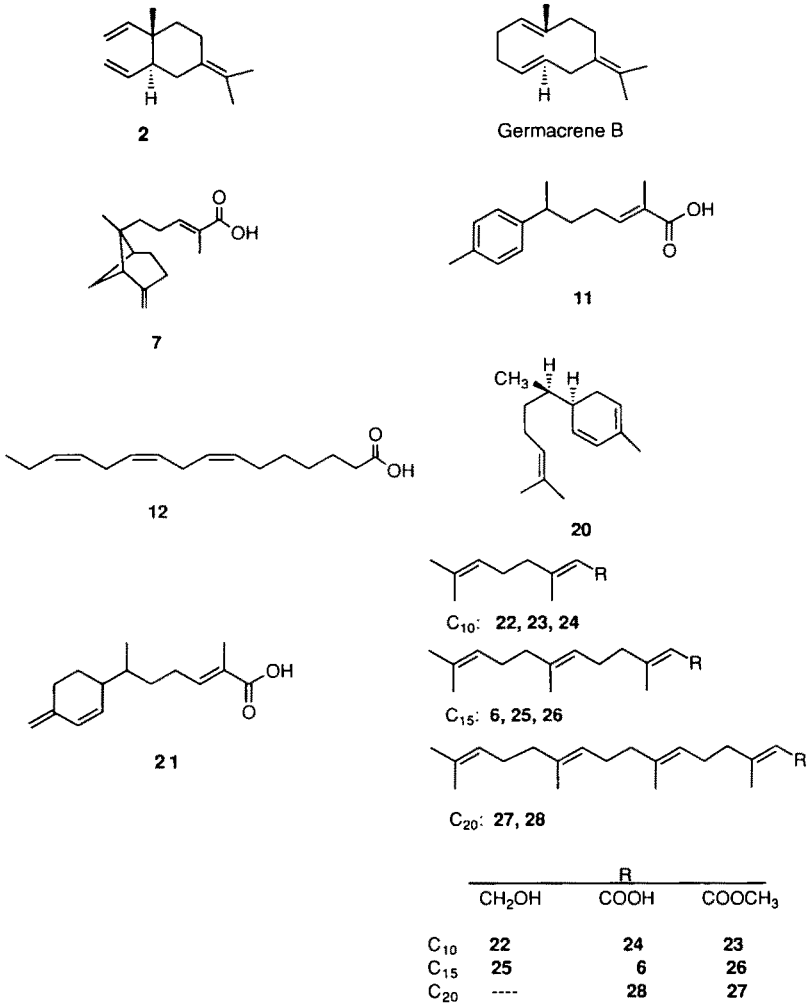

FIG. 1. *Helicoverpa zea* kairomones from Solanaceae species.

(**1**), 3-methylhentriacontane (**3**), *n*-dotriacontane (**4**), 2-methyltriacontane (**5**), 2-methyloctacosane (**9**), *n*-pentacosane (**10**), 3-methylnonacosane (**14**), 2-methylnonacosane (**16**). Wax fractions were separated from the rest of the neutral fraction by flash chromatography on silica gel, as described by Still (1978). The structure of these alkane waxes, as well as several others without apparent biological activity, were determined by the mass spectral methods discussed above. The MS for **1** is consistent with that in Gülz (1968); that for **3** is consistent with the data given in Brown et al. (1990); that for **5** is consistent with data in Pomonis (1989); those for **9** and **14** are consistent with spectra in Coudron and Nelson (1978). Alkanes **4** and **10** were identified by GC-MS comparisons (coelution and MS) with authentic standards (Sigma).

Germacrene B. This sesquiterpene was isolated from the neutral fraction of the hexane extract of *Lycopersicon hirsutum* LA 1557. The hexane extract was washed with aqueous 10% Na_2CO_3 to remove organic acids. Flash chromatography of the neutral fraction on 15% (w/w) silver nitrate silica gel with pure hexane as eluant provided a clear oil, GC analysis of which (at an injector temperature of 210°C) gave only one peak at 8.58 min. The ^1H NMR (400 MHz) spectrum of this oil in C_6D_6 (to avoid acid-catalyzed cyclization) provided a spectrum identical to that in the literature (Clark et al., 1987). ^{13}C NMR analysis gave the following resonances (100 MHz, C_6D_6): 14.26, 16.35, 20.47, 20.80, 22.70, 26.14, 32.87, 34.41, 39.25, 126.82, 127.88, 128.12, 128.42, 128.50, 131.55. GC reanalysis of this oil from LA 1557 with an injector temperature of 325°C, the temperature used for the GC analysis that provided the data for the statistical tests, showed peaks at 7.09 min (33%) and at 8.58 min (67%, germacrene B) with HR-MS obs. 204.1858, calc. for $C_{15}H_{24}$, 204.1878. In the original chromatograms of all the accessions, the occurrence and relative amounts of the chemicals with these two retention times was highly correlated ($R = 0.94$), as they would be if the two compounds were biochemically related or if one were a degradation product of the other. Unfortunately, our regression analysis implicated the earlier retention time, 7.09 min, which we believe to be that for γ-elemene (**2**), the Cope rearrangement product of germacrene B, as that of the biologically active product. This compound has previously been identified by MS and infrared spectroscopy from hexane washes of *Lycopersicon hirsutum* (Weston et al., 1989). We have not yet isolated γ-elemene (**2**) or germacrene B in quantities sufficient for bioassay.

(+)-(E)-endo-β-Bergamotenoic acid (7). The sesquiterpene acid was isolated from the hexane wash of the leaves of *Lycopersicon hirsutum* accession LA 1557 by means of base extraction, esterification, chromatographic fractionation, and saponification as described in Coates et al. (1988).

Palmitic Acid (18). This fatty acid, with HR-MS obs. 256.2411, calc. for $C_{16}H_{32}O_2$, 256.2402, was identified from *Lycopersicon hirsutum* f. *glabratum*

accession PI 126449 by computerized searching of a mass spectrum library, and the identity was confirmed by GC coinjection with an authentic sample (Aldrich) and comparison of its mass spectrum with that from the authentic sample. Samples used for bioassay were from Aldrich (99% purity).

(E,E)-*Farnesoic acid (6).* This compound was isolated by base extraction of the hexane wash from *Lycopersicon hirsutum* f. *glabratum* accession PI 199381, esterification with diazomethane, and subsequent chromatographic fractionation on silica gel and then 20% (w/w) silver nitrate–silica gel to obtain 167 mg of methyl farnesoate (83% purity by GC). HR-MS obs. 236.1771, calc. for $C_{15}H_{24}O_2$, 236.1776. The ^1H NMR shifts of the methyl ester compare closely with those in Kulkarni et al. (1987). The preliminary identification was confirmed by synthesis of methyl farnesoate from farnesol by Corey's method (Corey et al., 1968a,b) and GC coinjection and comparison of ^1H and ^{13}C NMR resonances of the synthesized sample and the methyl ester of **6.** ^{13}C NMR of the methyl ester gave the following shifts (100 MHz, $CDCl_3$): 15.89, 17.56, 18.70, 25.56, 25.83, 26.55, 39.56, 40.83, 50.63, 115.12, 122.75, 124.12, 131.25, 136.02, 160.04, 167.11. Saponification of 45 mg of the ester in 1 ml of absolute ethanol with 0.75 ml of aqueous 2 M KOH gave 20 mg of farnesoic acid (76% total acid purity by GC, with 64% 2*E*,6*E* isomer and 12% 2*Z*,6*E* isomer) for bioassay.

(R)-*2-Methyl-6-(4-methylphenyl)-2-heptanoic Acid (Curcumenoic Acid, 11).* This sesquiterpene acid (HR-MS obs. 232.1461, calc. for $C_{15}H_{20}O_2$, 232.1463) was isolated as its methyl ester from base extraction of the hexane wash of *Lycopersicon hirsutum* f. *glabratum* accession PI 199381. Esterification with diazomethane and subsequent chromatographic fractionation first on silica gel and then on 15% (w/w) silver nitrate–silica kgel gave 51 mg of the methyl ester of **11** (95% purity by GC). Saponification of 26 mg of the methyl ester (95% purity) by the method described above for **6** provided 18 mg of **11** (91% total acid purity, with 83% 2*E* isomer and 8% 2*Z* isomer, and an additional 5% of unreacted acid methyl ester). ^1H NMR of the methyl ester (400 MHz, $CDCl_3$): δ 1.23 (d, J = 7 Hz, 3 H), 1.69 (dt, J = 7.5, 1.8 Hz, 2H), 1.72 (d, J = 0.8 Hz, 3H), 2.04 (q, J = 3.1 Hz, 2H), 2.31 (s, 3H), 2.66 (sextet, J = 7.0 Hz, 1H), 3.71 (s, 3H), 6.73 (dt, J = 7.3 Hz, 1H), 7.08 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): 12.32, 20.96, 22.54, 26.79, 36.92, 39.07, 51.63, 126.80 (two overlapping peaks), 127.42, 129.04 (two overlapping peaks), 135.44, 142.46, 143.63, 168.65. The ^1H NMR data for the ester compare closely to those for (±)-methyl curcumenoate in Alexander and Rao (1968). Stereochemistry was determined by optical rotation (Breeden and Coates, 1994).

(Z,Z,Z)*Hexadeca-7,10,13-trienoic Acid (12).* This acid (HR-MS obs. 250.1932, calc. for $C_{16}H_{26}O_2$, 250.1933) was isolated as a mixture of its methyl ester with the methyl ester of linolenic acid. Base extraction of the hexane wash

of *Lycopersicon hirsutum* f. *glabratum* accession PI 199381, esterification with diazomethane, and subsequent flash chromatographic fractionation on silica gel and then on 15% (w/w) silver nitrate–silica gel afforded an oil that was 25% of the methyl ester of **12** and 75% of the methyl ester of linolenic acid (**19**), whose identification is discussed below. Further flash chromatography provided no further separation of the two compounds. The MS for **12** proved to be analogous to that for authentic linolenic acid (Sigma), with similar patterns of fragmentation and base peaks, except the mass peaks for **12** were consistently 28 atomic mass units fewer than those for linolenic acid. The ^{1}H NMR spectrum of the mixture of acid esters is identical to that for 96% pure linolenic acid methyl ester in location of shifts, peak size, and peak shape. ^{1}H NMR spectral values agree well with those predicted by the rules given in Frost and Barzilay (1971) and rule out *trans* or conjugated double bonds.

Some compounds were not implicated by the regression analysis as biologically active.

Linolenic Acid (19). This fatty acid, MS molecular weight 278, was identified by GC coinjection with authentic sample (Sigma). MS for the authentic sample and the sample from *Lycopersicon hirsutum* accession LA 1557 were identical. Samples used for bioassay were from Sigma (98% purity). ^{1}H NMR spectral values agree well with those predicted by the rules given in Frost and Barzilay (1971).

7-Epizingiberene (20). This sesquiterpene hydrocarbon (HR-MS obs. 204.1872, calc. for $C_{15}H_{24}$, 204.1878) has not been previously reported in the literature in this stereochemical form. It was isolated by flash chromatography of the neutral fraction of the *Lycopersicon hirsutum* f. *glabratum* accession PI 199381 hexane leaf wash. The regression analysis did not implicate it as stimulating oviposition. A full account of its isolation and identification is available in Breeden and Coates (1994).

Kairomone Oviposition Bioassays

Oviposition assays were conducted as described above, except that hexane solutions of the putative kairomones (concentrations of these solutions are described below) were used instead of crude plant extracts. The amounts of the kairomones to be tested were determined by comparison of their GC peak areas with that for β-bergamotenoic acid. For each of the naturally occurring compounds, the concentration on the leaf surface was determined by comparison of the area under the GC peak for that compound with that for β-bergamotenoic acid, for which a standardized amount per square centimeter of leaf area had already been determined (0.48 mg/cm^2) (Douglass et al., 1993). Compounds that were not isolated from the plants were applied on an equimolar basis (2.1

$\mu M/cm^2$) with the naturally occurring concentration of β-bergamotenoic acid (**7**).

Bioassay 1 tested straight-chain acids having chain length of 6 and between 8 and 18 carbons and was conducted to ascertain the relationship between the size of the acid molecule and moth oviposition chemoreceptor. The amounts tested were equimolar to **7** and were 4.31 mg/disk for octanoic acid, 4.72 mg/disk for nonanoic acid, 5.14 mg/disk for decanoic acid, 5.56 mg/disk for undecanoic acid, 5.98 mg/disk for dodecanoic acid, 6.40 mg/disk for tridecanoic acid, 6.82 mg/disk for tetradecanoic acid, 7.24 mg/disk for pentadecanoic acid, 7.65 mg/disk for hexadecanoic acid, and 8.49 mg/disk for octadecanoic acid.

Bioassay 2 tested the moths' response to the alkane wax fractions from several *Lycopersicon* accessions, in order to address the role of these compounds in *H. zea* oviposition. The amount (milligrams per square centimeter) of alkanes naturally occurring on the leaves was calculated, and because these concentrations were low, twice this amount was applied to the disks for bioassay. The alkane fractions were separated from the rest of the neutral fraction of each accession and suspended in hexane. For LA 1374, this was 0.30 mg/disk of total alkane fraction; for LA 1557, 3.96 mg/disk; for PI 126449, 3.47 mg/disk; for PI 365906, 2.85 mg/disk; for PI 365907, 0.75 mg/disk; for PI 365908, 0.66 mg/disk; for PI 390514, 0.58 mg/disk; and for PI 199381, 0.54 mg/disk.

Bioassay 3 tested the organic acid kairomones whose GC retention times had been implicated as biologically active. For this bioassay, the naturally occurring concentrations of each acid on the leaf surface (milligrams per square centimeter) were determined by comparison of the standardized GC peak area for that acid with the standardized GC peak area for β-bergamotenoic acid, whose leaf surface concentration was known. These amounts were then multiplied by the size of the disk, and then corrected for purity (as determined by GC). This amounted to 0.214 mg/disk for **12,** 1.46 mg/disk for **11,** 2.78 mg/disk for **6,** 0.12 mg/disk for **18,** 7.0 mg/disk for **7,** and 1.43 mg/disk for **19.** Because **12** was not chromatographically separable from **19,** a mixture of 75% **19** and 25% **12** was used, with the applied concentration of **12** being 0.214 mg/disk.

RESULTS

Accession Oviposition and Larval Orientation. GC data were compared with oviposition and larval preferences in order to show that the compounds played a role in stimulating oviposition and in larval preference. Analysis of variance revealed highly significant differences among the extracts for oviposition stimulation ($F = 5.40$, $P < 0.0001$ for 1988; $F = 7.71$, $P < 0.0001$ for

1989) and larval orientation preference ($F = 4.89$, $P < 0.0001$). The range of oviposition values for accessions from each of the species assayed is listed in Table 1. A complete list of these values for all accessions and chromatographic peak data is available upon written request to the corresponding author. The wide range of responses to the extracts suggested that certain plant species may be preferred over others in part because of their leaf surface constituents. These results indicated that extracts from members of the *Lycopersicon* and *Nicotiana* species had the greatest oviposition stimulation, that *Glycine max* had a strong stimulatory effect, and that other species, such as those of the genus *Gossypium*, had some stimulatory effect. This is in agreement with the work of Johnson et al. (1986), who reported a decreasing order of preference with corn > tobacco > soybeans > cotton for *H. zea* oviposition. In particular, wild tomato species had a strong stimulatory effect with six of the top 12 highest extract oviposition preference values.

Identification of Kairomones. Putative kairomones were implicated by the initial correlations, and then multiple regression was performed on a subset of the data, in order to select compounds for chemical isolation, purification, and identification. Table 2 lists the compounds suggested as biologically active, as well as the empirical formulas for those compounds, the number of accessions that had a compound with the same retention time, and the R^2 and P values for the correlations between standardized peak area of each putatively active component and oviposition or larval orientation values. The empirical formulas indicated that all the stimulatory compounds fell into the following chemical classes in order of their GC retention times: sesquiterpene hydrocarbons (**2**), sesquiterpene acids (**6, 7, 11**), fatty acids (**12, 18**), and long-chain alkanes (**1, 3, 4, 5, 9, 10, 14, 16**). Four of the compounds (**8, 13, 15, 17**) were not present in sufficient quantities to allow us to perform GC-MS on them, so no chemical data are available and they are listed as unknowns.

Simple correlations of moth oviposition preferences with larval orientation for 156 of the accessions planted in 1988 gave no correlation ($r = -0.09$, $P = 0.27$), which suggested that the compounds that stimulate oviposition for the moths did not influence orientation by the larvae. This may have been due to the reduced importance of chemoreception for larval feeding selection or, more likely, the fact that the larval chemoreceptors are sensitive to compounds found inside the leaf rather than those found only on the surface. An evolutionary scenario that might explain this is that the moths' chemoreceptors have been selected so as to be sensitive to compounds on the leaf surface that alert the moth to the presence of healthy, robust host plants on which to oviposit. In contrast, larval chemoreceptors more likely have evolved to be sensitive to compounds encountered during feeding.

Previous studies help to provide support for our results since the statistical analysis picked out compound **7**, which we know to be β-bergamotenoic acid

TABLE 2. EMPIRICAL FORMULAS, ABUNDANCE, AND STATISTICAL SIGNIFICANCE OF BIOLOGICAL ACTIVITY OF *Helicoverpa zea* MOTH AND LARVAL KAIROMONES

Biological activity[a]	Compound number	Compound name	Empirical formula	Number of accessions[b]	Simple regression	
					R^2 value	P value
Moth oviposition 1988	1	2-Methyldotriacontane	$C_{33}H_{68}$	66	0.1551	0.0008
	2	γ-Elemene	$C_{15}H_{24}$	21	0.3685	0.0035
	3	3-methylhentriacontane	$C_{32}H_{66}$	120	0.0732	0.0023
	4	*n*-Dotriacontane	$C_{32}H_{66}$	125	0.2002	0.0000
	5	2-Methyltriacontane	$C_{31}H_{64}$	121	0.1862	0.0000
	6	(*E*,*E*)-Farnesoic acid	$C_{15}H_{24}O_2$	4	0.8931	0.0045
	7	β-Bergamotenoic acid	$C_{15}H_{22}O_2$	8	0.9142	0.0001
Moth oviposition 1989	8	Unknown	unknown	6	0.6883	0.0411
	9	2-Methyloctacosane	$C_{29}H_{60}$	3	0.8849	0.0172
	10	*n*-Pentacosane	$C_{25}H_{52}$	7	0.7990	0.0066
	11	Curcumenoic acid	$C_{15}H_{20}O_2$	11	0.7605	0.0005
	12	7,10,13-Hexadecatrienoic acid	$C_{16}H_{26}O_2$	81	0.1986	0.0000
Larval preference 1988	13	Unknown	unknown	52	0.1279	0.0092
	14	3-Methylnonacosane	$C_{30}H_{62}$	36	0.2755	0.0010
	15	Unknown	unknown	3	0.6284	0.0189
	16	2-Methylnonacosane	$C_{30}H_{62}$	7	0.5037	0.0740
	17	Unknown	unknown	15	0.4164	0.0038
	18	Palmitic acid	$C_{16}H_{32}O_2$	59	0.0463	0.0780

[a] Biological activity indicates the type of activity with which the compound was associated. Because the GC retention times for 1988 and 1989 were incompatible, statistical correlations were run separately for each year.

[b] The number of accessions listed is the number of accessions that had a GC peak at the retention time for that compound, out of 226 total accessions tested in 1988 and 96 accessions tested in 1989.

(Coates et al., 1988; Juvik et al., 1988), from the 310 compounds present in the accessions tested in 1988. That this stimulatory compound, present in only eight accessions, was implicated as active by the regression analysis validates this procedure. All of the compounds implicated by our regressions as having biological activity were later observed in their isolated forms to confer *H. zea* oviposition stimulation. Other compounds not quantitatively associated with oviposition preference in our observations (e.g., palmitic and linolenic acids, **18** and **19**; see Table 4 below) were inactive when later isolated and bioassayed.

The empirical formulas of the compounds associated with biological activity suggested that only five of the 18 chemicals so implicated are organic acids. The acids may have played a more specialized role in stimulating oviposition than previously reported. Most of the active compounds were saturated long chain alkanes. Eight of the 18 compounds contributing to the R^2 value for 1988 oviposition equations were alkanes. The differences in structure and electron densities between long-chain alkanes and organic acids further suggested that at least two different sets of receptors that mediate oviposition response may exist in *H. zea*, since the shape and electronic properties of these two classes would likely produce distinct interactions with the receptors (Moncrieff, 1967). The empirical formula of the remaining compound suggests that it is a sesquiterpene alkene. This may represent a third chemical class of oviposition stimulants for *H. zea*.

TABLE 3. *Helicoverpa zea* OVIPOSITION PREFERENCES FOR ALKANE WAX FRACTIONS FROM *Lycopersicon hirsutum* ACCESSIONS

Accession name	Amount applied (mg)[a]	Oviposition preference[b]
Hexane control	0.0	0.965a
Lycopersicon hirsutum, LA 1374	0.30	1.002a
Lycopersicon hirsutum f. *glabratum*, PI 365908	0.66	1.060a
Lycopersicon hirsutum f. *glabratum*, PI 365907	0.75	1.008a
Lycopersicon hirsutum, PI 365906	2.85	1.462b
Lycopersicon hirsutum f. *glabratum*, PI 390514	0.58	1.537b
Lycopersicon hirsutum f. *glabratum*, PI 199381	0.54	1.570b
Lycopersicon hirsutum f. *glabratum*, PI 126449	3.47	2.082c
Lycopersicon hirsutum, LA 1557	3.96	2.408d

[a]The amount applied is twice the naturally occurring concentration.

[b]Oviposition preferences were calculated by dividing the number of eggs on the disk on a given day by the mean number of eggs on two adjacent untreated disks and are reported as means over three days and four replications. Means followed by dissimilar letters are significantly different at $P = 0.05$ using Fisher's protected LSD test.

The results of bioassay 2 are given in Table 3 and indicated significant ($P < 0.05$) differences among oviposition preferences for the alkane wax fractions of the accessions listed. These differences may be explained largely as a function of overall alkane concentration, because regression of the summed peak areas of the alkanes against the oviposition preference values for each accession yielded an r value of 0.86, with $P = 0.001$. Six alkanes of the 13 analyzed by multiple regression were identified as significantly contributing to the model R^2 value, and these accounted for 100% of the variation in the model. When these vari-

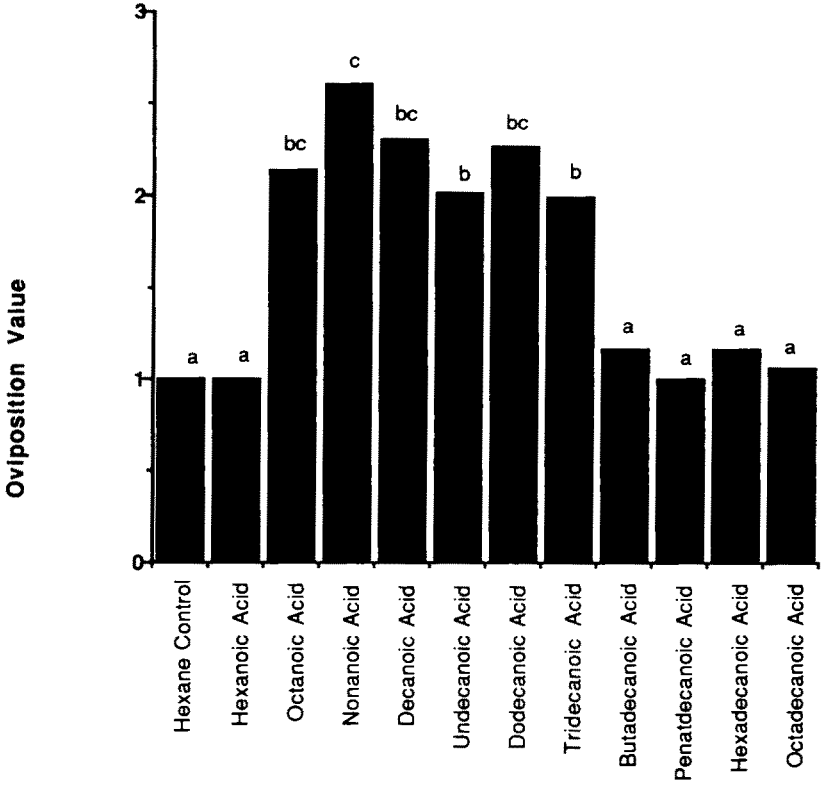

FIG. 2. *Helicoverpa zea* preference values, as a function of chain length of aliphatic organic acids. Acids were applied in equimolar amounts. Oviposition preference values were calculated by dividing the number of eggs on a treated disk by the mean number of eggs on two adjacent untreated disks and are reported as means over two days and four replicates. Dissimilar letters indicate significant differences between means, using Fisher's protected LSD test ($P = 0.05$).

ables were removed and multiple regression was rerun on the remaining waxes, the remaining seven variables still accounted for 90% of the total variation. This suggested that the moths do not discriminate between individual alkanes and that most or all of the alkanes stimulated oviposition to some extent. Moth perception of the waxes may be a response to the physical texture of the epicuticular surface elicited by tarsal mechanoreceptors in contrast to chemical reception.

The results of the aliphatic acid oviposition bioassay (bioassay 1), presented in Figure 2, showed significant ($P < 0.05$) differences among the acids tested. Only acids with greater than seven and fewer than 14 carbons significantly stimulated oviposition. Douglas et al. (1993) also reported that heptanoic and octanoic acids significantly stimulated *H. zea* oviposition while hexanoate did not. This is in agreement with the hypothesis that the shape of the molecule determined the ability of the kairomone to bind to the receptor, as those beyond a certain length did not stimulate oviposition, nor did those with fewer than seven carbons. It further helped to confirm our suggestion that the moth responded more to the acid side chain of β-bergamotenoic acid than to the bicyclic ring (Douglass et al., 1993).

The results of bioassay 3 of six naturally occurring acids, of which four had been implicated as stimulating oviposition (**12, 11, 7, 6**), are given in Table 4. All four of the acids that had been implicated as stimulating oviposition were significantly active. Neither linolenic (**19**) nor palmitic acid (**18**), which were present in many of the extracts analyzed, but not implicated as active by regression analyses, was found to stimulate oviposition. These results again validated

TABLE 4. *Helicoverpa zea* OVIPOSITION PREFERENCES FOR KAIROMONAL ACIDS ISOLATED FROM *Solanaceae* SPECIES

Compound name	Compound number	Amount applied (mg)	Oviposition preference[a]
Hexane control	—	0.0	0.972a
Palmitic acid	18	0.12	1.092a
Linolenic acid	19	1.43	2.030ab
Hexadecatrienoic acid	12	0.214	2.590bc
Curcumenoic acid	11	1.46	3.205c
β-Bergamotenoic acid	7	7.00	9.788d
Farnesoic acid	6	2.78	12.802d

[a]Oviposition preferences were calculated by dividing the number of eggs on the disk on a given day by the mean of the number of eggs on the two adjacent untreated disks and are reported as means over three days and four replications. Means followed by dissimilar letters are significantly different at $P = 0.01$ using Fisher's protected LSD test.

the regression procedure for correctly implicating compounds that contribute to oviposition stimulation, since compounds that were present in only a very few accessions, like farnesoic acid (**6,** four accessions), and curcumenoic acid (**11,** 11 accessions), were implicated. The statistical technique also implicated stimulatory compounds that were present in only very small quantities, such as 7,10,13-hexadecatrienoic acid, which was found in 82% of the accessions tested in 1989, but accounted for (on average) only 2.0% of the total peak area from the chromatograms of the extracts.

DISCUSSION

None of the compounds in the extracts was observed to be associated with reduced oviposition. This lack of moth repellents/oviposition deterrents from any of the tested accessions suggests that *H. zea*'s perception of host-plant chemical cues only elicit positive ovipositional responses. This is in keeping with the species' polyphagous nature and reproductive strategy. It would also allow for ovipositional "mistakes" that would diversify *H. zea*'s host range, provide the opportunity to adapt to agricultural monocultures, and potentially result in subspeciation.

That kairomones and allomones influence insect behavior is well established in the literature (Juniper and Southwood, 1986; Rosenthal and Berenbaum, 1991), and evidence suggests that these chemicals influence behavior through their interaction with specifically shaped protein-based chemoreceptors (Moncrieff, 1967; Evershed, 1988). A molecule of the appropriate size and shape with charge densities in the appropriate orientation will interact with the chemoreceptor and elicit a neurochemical response, whereas other molecules will not. Slight modification of the molecular structure of a stimulant may abolish its biological activity entirely, as in the case of *Bactrocera dorsalis* (Metcalf et al., 1979, 1981, 1983). Stimulation of feeding by *B. dorsalis* was reported by Metcalf et al. (1981) to be associated with hydrophobicity and the location of electron rich areas in various compounds that they synthesized and bioassayed.

Douglass et al. (1993) tested various bicyclic and acyclic analogs of β-bergamotenoic acid on *H. zea* oviposition preference. They proposed that the hydrogen bonding between the $-$OH functionality of the carboxylic acid group and the chemoreceptor plays a crucial role in biological activity, because while cyclic and acyclic acids stimulated oviposition, esters, alkenes, and acetates did not. They also found that modifying β-bergamotenoic acid by reducing the side-chain double bond (thereby allowing free rotation of the acid group) increased the oviposition response as well.

Straight-chain acids have been reported to be ubiquitous (Kolattukudy,

1969; Tulloch, 1976), and the oviposition activity of these acids may help to explain the evolutionary significance of the relationship between *H. zea* and β-bergamotenoic acid. Because β-bergamotenoic acid is so rare (present in only eight accessions), it seems odd that it would be a primary stimulant for a moth with as wide a host range as *H. zea*. If, however, as Douglass et al. suggest, the stimulatory power of this rare acid developed as a coincidence of the stimulatory power of the much more common aliphatic acids, then the evolutionary picture is clearer.

If the compounds identified in this paper as long-chain alkane waxes play a role in oviposition stimulation, this may help explain the polyphagy of *H. zea*, as these compounds are ubiquitous (Kolattukudy et al., 1976). Epicuticular waxes have been shown to stimulate oviposition in several insect species (See review by Eigenbrode and Espelie, 1995). These compounds serve the plant by performing such functions as water repellency and retention, frost resistance, transpiration regulation, etc. (Tulloch, 1976; Jeffree, 1986). Alkane waxes would be ideal host indicators for phytophagous insects, because compounds as important as these to the plants' biology would likely not be selected against in the course of evolution, regardless of their kairomonal properties for host species. Thus, the cycle of selection and adaptation described by Ehrlich and Raven (1964), in which the evolutionary advantage and opportunity for speciation switch from the insect to host species and back again, would be circumvented because of the advantage conferred by the alkanes regardless of their stimulatory role in *H. zea* oviposition.

Alkane waxes serve a number of roles in the ecology of *H. zea*. In addition to the biological functions mentioned above, Hendry et al. (1976) reported that four *n*-alkanes, which serve as kairomones for the *H. zea* parasite *Trichogramma evanescens*, constituted the major portion of the surface lipids of *Zea mays*, a host plant for *H. zea*. The incorporation of alkanes endogenous to plants into the cuticular lipids of insects feeding on the plants has been demonstrated (Espelie and Bernays, 1989; Blomquist and Jackson, 1973). If the alkanes from *Zea mays* are incorporated directly into *H. zea* from the plant source, rather than synthesized de novo or chemically modified after consumption, then, while they serve as kairomones for *T. evanescens*, they may also serve as allomones for the plant by attracting parasites of *H. zea*. Together, these reports suggest that alkanes may play a more significant role in the ecology of *H. zea* than might have been anticipated for such seemingly structurally simple compounds.

The activity of these alkanes is likely an adaptive response by the moth to plant leaf chemistry. The alkanes may serve as a nonspecific cue for living plants. The next step in *H. zea* host differentiation could then have been the evolution of moth perception of compounds with the acid moiety. The stimulatory power of these acids may represent an evolutionary event associated with

the speciation of *H. zea*, since oviposition by *H. virescens*, a sibling species, is stimulated by the long-chain alkanes but not by the acid compounds (Juvik, unpublished data). The structural dissimilarities between these two classes of compounds suggest that at least two distinct reception systems (one possibly mechanical and the other chemical) stimulate oviposition behavior in *H. zea*. The evolution of the acid chemoreceptor and its influence on host plant selection may have been associated with the phylogenetic divergence of *H. zea* and *H. virescens*.

Similarly, the results of the acid bioassays may help to explain *H. zea*'s polyphagy. Although the terpene acids are taxonomically restricted in the plant kingdom, the data indicate that the acid functionality of more common aliphatic acids (Kolattukudy et al., 1976) may also serve as stimulants. While the data in Tables 2 and 4 and in Figure 2 indicate that acids of the proper chain length stimulate oviposition, other functional groups [such as the side-chain methyl groups and sites of unsaturation in farnesoic acid (**6**)] also play a role. This sesquiterpene acid (**6**), even though present at only one third the molar concentration of β-bergamotenoic acid, was more stimulatory for *H. zea* oviposition activity. We propose that the active acids are stimulating the same receptor system, with the differences explicable on the basis of other functionalities among the molecules. The role of these other functionalities is also made evident by the slight stimulatory activity of 7,10,13-hexadecatrienoic acid, which has a chain length of 16 carbons and stimulated activity, while the saturated C_{16} palmitic acid did not. It is not clear from these assays whether this increased activity is attributable to the ability of the electron densities in the unsaturated acids to interact with chemoreceptors or to the fact that the *cis* double bonds in 7,10,13-hexadecatrienoic acid shorten the total length of the molecule such that it is more structurally compatible with the receptor. Molecular modeling could be used to determine whether the sites of unsaturation significantly shorten the molecule.

The sesquiterpene acids are also implicated in other ecological relationships. β-Bergamotenoic acid (**7**) has been implicated as an allelopathic compound, inhibiting seed germination and root formation in a number of different species, including Grand Rapids lettuce, purslane, wild mustard, and cultivated tomato (Bish et al., 1993). Thus, while this acid stimulates *H. zea* oviposition, it is evolutionarily advantageous in its allelopathic abilities. Similarly, curcumenoic acid (**11**), β-bergamotenoic acid (**7**), farnesoic acid (**6**), and γ-elemene (**2**), which strongly stimulate *H. zea* to oviposit, have also been reported as deterrents to the spider mite (*Tetranychus urtichae*), a pest of tomatoes (St. Pyrek et al., 1991; Weston et al., 1989; Guo et al., 1993). In addition, St. Pyrek and Snyder have shown that 2,3-dihydrofarnesoic acid and zingiberenoic acid confer resistance to *T. urtichae* (Snyder et al., 1993), and we have tenta-

tively identified both from the extract of *L. hirsutum* f. *glabratum* PI 199381 by MS and HR-MS. The allomonal properties of these compounds may also provide an evolutionary explanation for the production of these acids.

The identification and confirmation of the biological activity of these compounds may be useful both in pest management programs and more broadly in chemical ecology. As detailed in Metcalf (1985), there are a number of pest-control strategies that are connected with identification of kairomonal allelochemicals, including trapping for the purposes of population monitoring, removal trapping, and behavior disruption, as has been done for *Dacus cucurbitae* (Metcalf, 1985). Similarly, genetic manipulation could be used in the case of some of the sesquiterpenoic compounds, if not the alkanes, to produce plants without these cues and to induce antixenosis. Conversely, the cues could be bred into commercially less valuable crops for the purposes of providing a decoy to lure *H. zea* away from the protected crops, as has been successfully done by using *Curcubita ecuadorensis* as a trap for *Diabrotica speciosa* (Metcalf, 1985). These examples suggest that environmentally friendly agricultural practices that arise from ecological research may be viable on the scale needed for commercial production.

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ROLE OF PLANT VOLATILES IN THE SEARCH FOR A HOST BY PARASITOID *Diglyphus isaea* (HYMENOPTERA: EULOPHIDAE)

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Abstract—*Diglyphus isaea* Walker is a larval ectoparasitoid used in biological pest control against the American serpentine leaf miner *Liriomyza trifolii* Burgess. We studied the parasitoid's host searching behavior, using olfactometric methods. Our data show that the parasitoids locate host larvae (a leaf-mining dipteran) on the basis of volatile signals released by the plant-host complex. Female *D. isaea* are strongly attracted to the odors arising from damaged bean plants, whereas they show practically no response to intact plants. The results of our chemical analyses showed that about 15 components were present, two of which, *cis*-3-hexen-1-ol and 4-hydroxy-4-methyl-2-pentanone, were present in significantly larger quantities in the leaf extracts from mined or damaged bean plants than in those from healthy plants. The damage inflicted by the host larvae on these plants triggers the release of larger amounts of these substances, which probably lead the parasites to their hosts. The compounds thus act as synomones.

Key Words—Parasites, volatile signals, synomone, host detection, Hymenoptera, Eulophidae, *Diglyphus isaea*.

INTRODUCTION

Chemical signals play an important role in the interactions between plants, phytophagous insects, and the latter's natural enemies. When searching for their hosts, numerous entomophagous parasitoids rely on volatile chemical stimuli

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arising from their host's natural habitat (Vinson, 1975, 1981; Weseloh, 1981; Van Alphen and Vet, 1986). Once the host's habitat has been located, the parasitoids have to find the hosts themselves. For this to be possible, the parasitoids have to be able to detect their hosts from a distance and to orient their movements in response to stimuli produced or induced by the host larvae or by their activity on the plant (Weseloh, 1981). The natural enemies of several phytophagous insects are strongly attracted by volatile compounds released by the host plants when they are damaged (Drost et al., 1988; Dicke et al., 1990; Turlings et al., 1990, 1991). The components of the plant–host complex may originate from either the plant, the host insect, or from the latter's feces or saliva (Vinson, 1976; Kennedy, 1984; Mudd et al., 1984; Takabayashi and Takahashi, 1989).

In order to understand insect behavior involving chemical communications, it is necessary to determine the nature of the signals arising from the insect's natural environment, that play an important role in their intra- and interspecies relationships. Chemical signals are known to play a role in *Dacnusa sibirica* (Dicke and Minkenberg, 1991) and in *Opius dissitus* (Petitt et al., 1992). In the present study, we analyzed the chemical stimuli used by the entomophagous parasitoid, *Diglyphus isaea* Walker (family Eulophidae). *D. isaea* is used in biological control of agromyzid leaf miners in greenhouses (Lyon, 1986; Nedstam et al., 1987; Van Lenteren and Woets, 1988). The ectoparasitoid uses several leaf miners as its hosts, especially *Liriomyza trifolii*, a pest that inflicts considerable damage on greenhouse vegetable and flower crops. Female parasitoids oviposit in the last larval stages of *L. trifolii* (Minkenberg and Van Lenteren, 1987).

More specifically, the aim of the study was to investigate whether *D. isaea* uses volatile chemicals to locate its hosts, and if so, to determine which of the chemical signals emitted by the plants, the agromyzid larva, and larval secretions (saliva, feces) influence the behavior of the parasitoids.

METHODS AND MATERIALS

Insects. The parasitoids used in these experiments were from a permanent rearing station set up by the INRA at Antibes, using the "alternate open field rearing and caged ovipositing" method (Lyon, 1976).

Plants. Bean plants (*Phaseolus vulgaris*) were grown in a nursery garden and were harvested at the cotyledon stage.

Behavioral Analyses. Behavioral observations were conducted with 2-day-old female *D. isaea* in their rearing cages (50 × 50 × 60 cm). For each experiment, two bean plants, each in its own pot, were provided; one plant contained only two healthy leaves and the other, two leaves mined by stage 2

(L2) larvae. Five female parasitoids were used in each test and 15 replications were conducted. Parasitoid visits to each of the two kinds of leaves were counted during a period of 20 min. Tests were carried out at 25°C and 50% relative humidity.

The Olfactometer. The linear olfactometer was a combination between the olfactometer designed by Leconte and Thibout (1981) and that described by Zagatti (1985). It was 30 cm in length, 2 cm in diameter, and was divided into compartments numbered 0–5 through which pure (control) air and air containing the odor to be tested were pushed alternately for 4 min. The device was placed between two neon-shelving (upper tube: 60 W, lower tube: 2 × 60 W). The parasitoids were classified as weakly attracted, moderately attracted, or strongly attracted depending on where they were located in the olfactometer. Five 2-day-old mated female parasitoids were placed in compartment 0 at the beginning of each test. All the tests were carried out at 25°C with 50% relative humidity. Circulating air (10 cm/sec) was filtered through an active charcoal disk and then through a tube containing fibreglass wool.

The odor streams were delivered as follows: for each test, 2 μl of the extract was placed on a 2-cm^2 filter paper dish and when the solvent had completely evaporated, the filter paper was placed in the olfactometer and swept by a stream of humidified air. The control airstreams were passed over a similar Whatman paper filter treated only with solvent.

Linear Olfactometer Data Processing. The number of females in each compartment was recorded every 30 sec, and 16 replications were conducted. These data were used to calculate the relative attraction indices as described by Zagatti (1985).

$$\frac{\sum_{i=0}^{5} in_{it} - \sum_{i=0}^{5} in_{i0}}{\sum_{i=0}^{5} (5 - i)n_{i0}} \times 100$$

where i is the number of compartments in the olfactometer (from 0 to 5), n_{it} is the number of insects in the compartment i at time t, and n_{i0} is the number of insects in the compartment i at $t = 0$. The results were analyzed statistically with the Mann-Whitney test.

Collecting and Analyzing Volatile Substances. A cold-trapping method was used to collect the odors arising from healthy beans, beans infested by stage two larvae, and beans that had been mechanically damaged by cutting the leaves with a razor blade. A steady flow (500 ml/min) of purified air was circulated through a closed 2-liters experimental chamber containing 25 leaves during 20 min. The molecules emitted were trapped on the walls of a capillary tube (10 cm × 0.2 mm) that was embedded in dry ice at −80°C. The trapped odors

were eluted with 300 ml of dichloromethane. The extract was then concentrated to obtain 50 μl and used in behavioral tests in the olfactometer and for chemical analyses. Samples were analyzed with a gas chromatograph, a Delsi 330 apparatus, equipped with a flame ionization detector and a Supelcowax 10 polar capillary column (30 m $\times$ 0.32 mm, 0.25-μm film thickness). The split/splitless injector was kept in the splitless mode for 15 sec after the injection. The carrier gas was helium at a pressure of 1 bar. The temperature program was isothermal at 30°C for 10 min and then increased in 5°C/min steps to 220°C. The data were captured and processed by a Chromatopac C-R4AX Shimadzu integrator. The results were quantified by introducing a solution containing two internal standards: 375 ng of *n*-dodecane and 400 ng of octanol.

Leaf extracts were obtained by soaking about 50 previously weighed leaves in 200 ml of dichloromethane for 24 hr. The extract was then filtered and concentrated in a nitrogen atmosphere. GC analyses confirmed that these samples contained the same substances as those obtained with the air-entrainment trapping method, but in higher concentrations. For identification of components, it was decided, therefore, to soak the leaves and to use gas chromatography coupled to mass spectrometry (GC-MS). The equipment consisted of a Hewlett-Packard 5890 Series II GC equipped with a single Supelcowax 10 polar column (30 m $\times$ 0.32 mm, with 0.25-μm film thickness), connected to a Hewlett-Packard 5989A MS Engine (EI, 70 eV). The apparatus was controlled by an MS Chemsystem HPUX multitask data processor using the Unix system. Helium at a pressure of 2 bars was used as the carrier gas. The GC oven temperature was maintained at 30°C for 5 min before being increased in 5°C/min steps to 210°C, and maintained for 10 min. The temperature was 240°C at the source, 250°C at the interface, and 100°C at the quadrupole. The masses were swept at 35–300 mass units at a rate of one scan per second. A standard range of *n*-alkanes from *n*-C_{12} to *n*-C_{20} was coinjected, and comparisons with the Wiley 138 library of reference spectra aided in identification of the main compounds present.

RESULTS

Behavioral Data. In the experimental cage, *D. isaea* made more frequent visits (Table 1) to mined leaves (43 visits) than to intact leaves (15 visits, $Z = 1.3 \times 10^{-4}$, $P < 0.001$). The parasitoid therefore appeared capable of distinguishing between the two categories of leaves. The mined leaves had yellowish tunnels that were clearly visible at the surface, each containing one larva. It is possible that the parasitoids located the mines through visual cues. In any case, the results of this test show that *D. isaea* are able to distinguish between healthy leaves and those containing a host larva.

TABLE 1. BEHAVIOR OF *Diglyphus isaea* RELEASED IN CAGE CONTAINING UNDAMAGED AND DAMAGED BEANS[a]

	Number of visits/20 min trial
Mined leaves	4 1 3 3 3 3 1 2 2 2 3 4 4 4 4 =43
Healthy leaves	1 2 0 1 0 2 1 1 1 0 1 3 1 0 1 =15

[a]The results from 15 replications were compared using the Mann-Whitney test. ($Z = -3.82$; $P < 0.001$).

The results of the linear olfactometer tests on female parasitoids exposed to trapped odors of healthy plant leaves are given in Figure 1. During the first 3 min, the females reacted very little to the trapped volatile substances originating from a healthy bean plant. The relative attraction indices (RAI) were low (5.6, 6.9, 7.4, 9.9, 9.5). Female parasitoids nevertheless showed a characteristic behavior in which they positioned themselves facing the oncoming flow of odor-

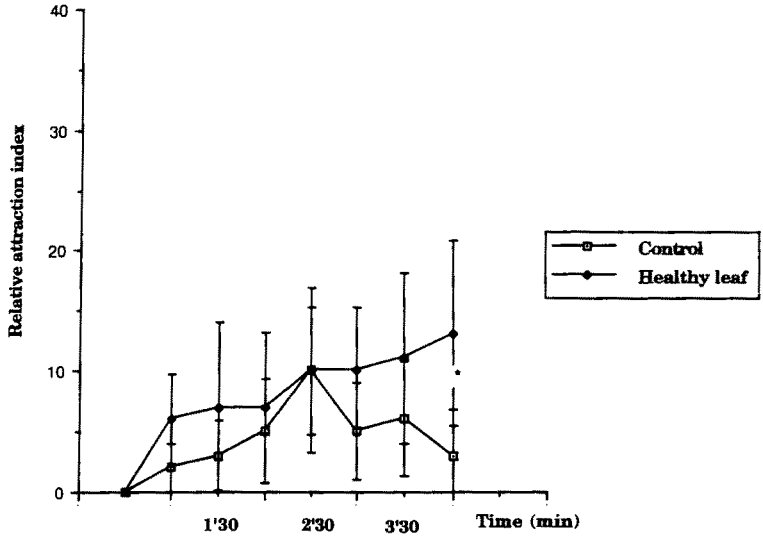

FIG. 1. Bioassay of healthy bean plant leaves and the control (pure air) stimulus for attraction of parasitoid females. There were 16 replications involving five females in each replication. Statistical analysis: Mann-Whitney test, $*P < 0.05$.

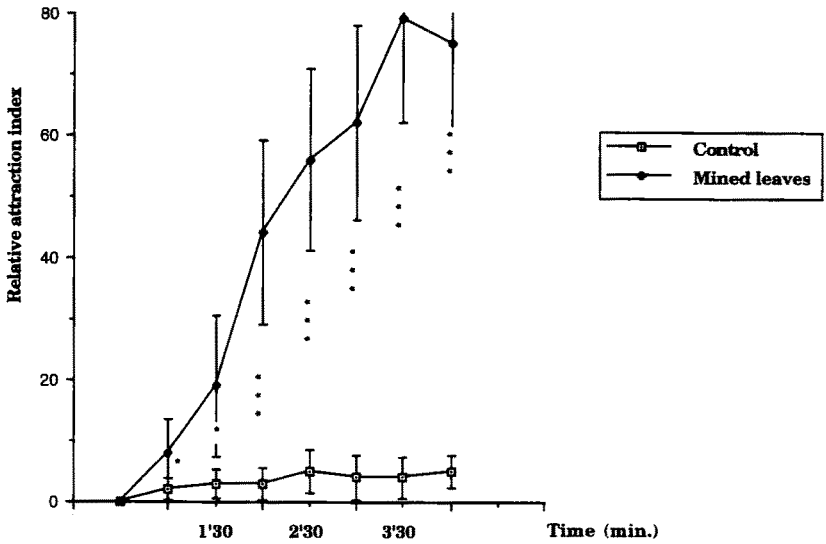

FIG. 2. Movement of five parasitoid females (16 replications) towards extracts of mined bean plant leaves compared with the control stimulus (pure air). Statistical analysis: Mann-Whitney test, $*P < 0.05$; $***P < 0.001$.

impregnated air with their antennae extended, and then made a series of antennal movements before advancing against the airstream. A significant difference was found to exist 4 min after the beginning of the experiments ($t + 4$ min) between the RAI indices obtained with impregnated and pure air at the 5% significance level ($Z = 0.25$) (RAI = 13.4 vs. RAI = 3.1 under control conditions).

In response to the airstream carrying trapped volatile substances extracted from bean plants infested with stage 2 larvae, female parasitoids oriented much more quickly towards the oncoming airstream (Figure 2). The RAI increased much more quickly, from 7.6 after 1 min to 61.7 after 3 min, and reached 75.5 by the last minute of observation. Females actively worked their way up against the odor-laden stream of air, frequently contracting their abdomen. This behavior is typical of females moving towards a mined area. As early as 1 min after the beginning of the test, the differences between the control test and the tests with extracts were significant at the 5% threshold level ($Z = 0.014$), and shortly afterwards, they became significant at the 1% level ($Z = 1.8 \times 10^{-6}$ at the fourth minute of the test).

Figure 3 gives the comparative data on movements of the female parasitoid towards healthy versus mined bean plants. These females also responded to the airstream carrying the trapped odor of mechanically damaged leaves (Figure 4).

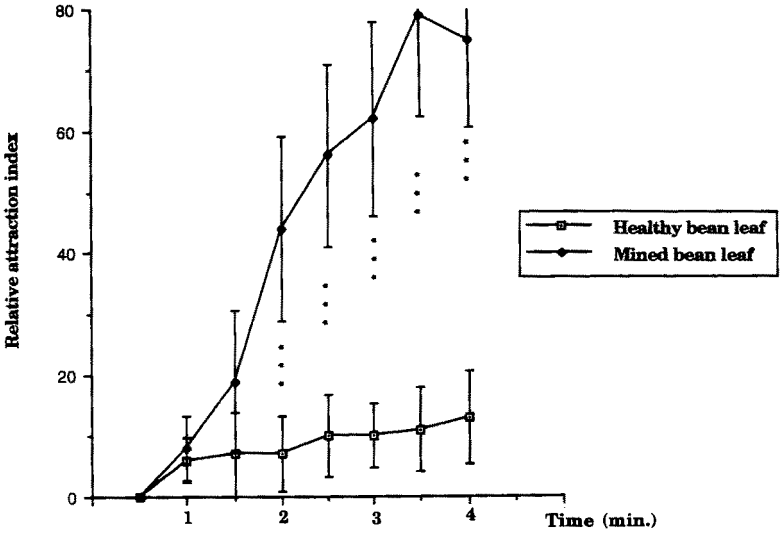

FIG. 3. Movement of parasitoid females (5 females, 16 replications) towards extracts of healthy bean leaves compared with mined bean leaves. Statistical analysis: Mann-Whitney test, $***P < 0.001$.

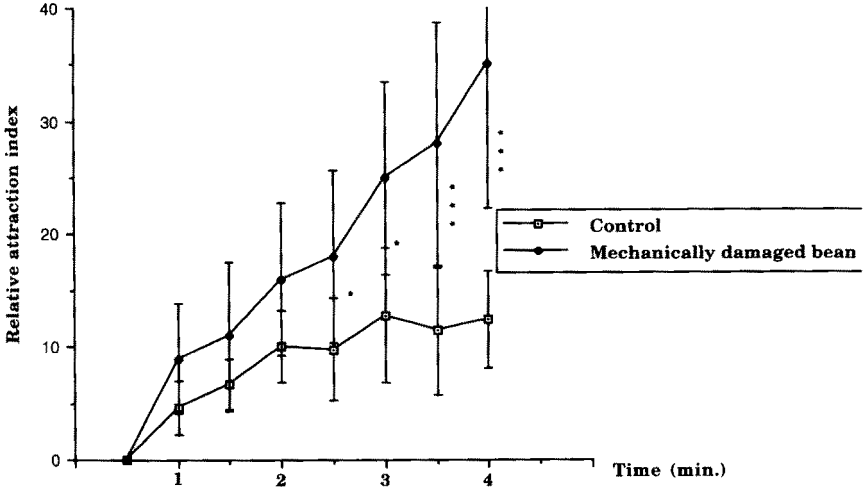

FIG. 4. Attraction of parasitoid females (5 females, 16 replications) by extracts of mechanically slashed bean plant leaves compared with the control stimulus (pure air). Statistical analysis: Mann-Whitney test, $*P < 0.05$; $***P < 0.001$.

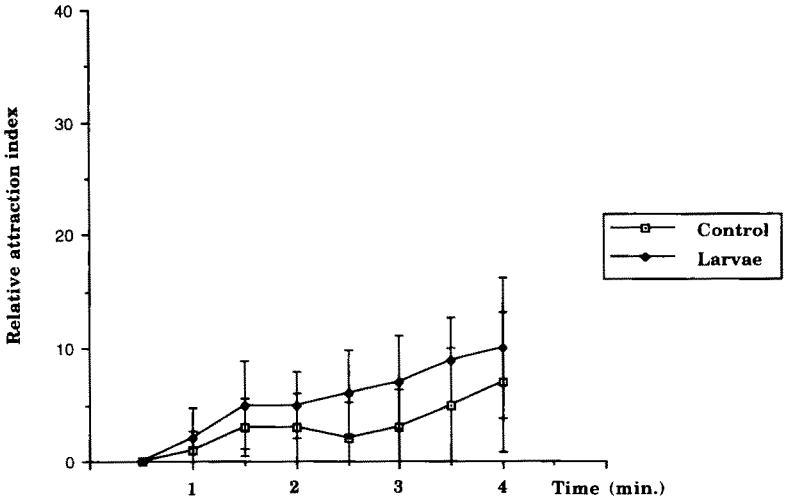

FIG. 5. Movement of parasitoid females (5 females, 16 replications) towards leaf miner larvae previously fed with bean plant leaves. Statistical analysis: Mann-Whitney test, not significant.

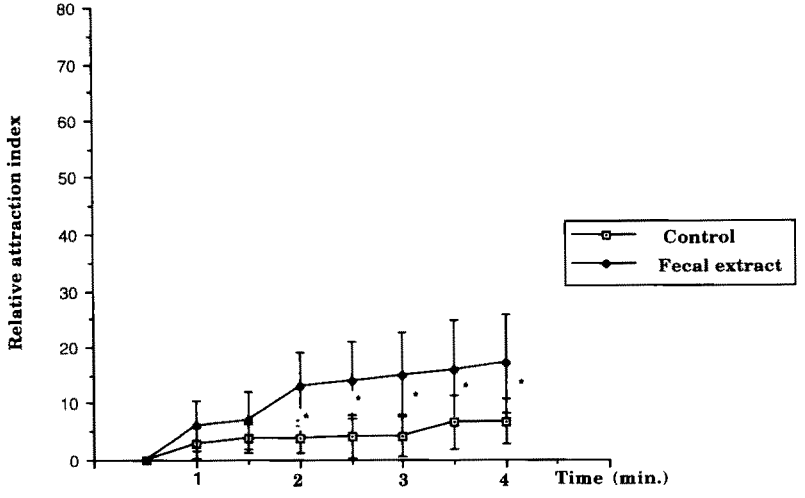

FIG. 6. Movement of parasitoid females (5 females, 16 replications) towards fecal extracts of leaf miner larvae compared with the control stimulus (pure air). Statistical analysis: Mann-Whitney test, $*P < 0.05$.

The attraction indices increased by 8.8 at $t + 1$ min, and reached 34.7 at $t + 4$ min. In comparison with pure air conditions, the RAI differed significantly at the 5% threshold ($Z = 0.025$) at $t + 3$ min, and then at the 1% threshold ($Z = 7.76 \times 10^{-3}$ at $t + 3.5$ minutes, $Z = 2.24 \times 10^{-3}$ at $t + 4$ min). Here the parasitoids' behavior was fairly similar to that observed with mined leaves.

The movements of *D. isaea* females towards stage 2 *Liriomyza trifolii* larvae did not differ from those elicited by control air (Figure 5). The relative attraction indices followed the same pattern in both cases, ranging between 2.2 and 10 with odor-impregnated air and between 1 and 7.2 with pure air ($Z = 0.82$ at $t + 1$ min and $Z = 0.5$ at $t + 5$ min). These data show that the host larvae alone exerted no attraction for the parasitoids.

The odor of the feces of leafminer larvae was attractive to the parasitoid females (Figure 6). The RAI reached 16.8 within $t + 4$ min, as compared with 6.7 in pure air ($Z = 0.06$). The differences observed between the indices recorded under control conditions and with the fecal odors were significant within 2 min at the 5% threshold level ($Z = 0.19$ at $t + 2$ min, $Z = 0.02$ at $t + 2.5$ min, $Z = 0.17$ at $t + 3$ min).

Chemical Analysis. Representative chromatograms obtained with trapped volatile extracts from healthy, mined, and mechanically damaged bean plant leaves are shown in Figure 7. Fifteen peaks occurred consistently from one test to another. None of these compounds was detected in the blank trapping extracts used as controls.

Figure 8 shows details of a typical reconstructed ion chromatogram obtained with mined leaves that were soaked in dichloromethane. Some of the substances were present only in trace quantities in the healthy leaves (peaks 7, 9, and 10). The composition of all substances present in larger amounts could be clearly determined. Three of the quantified components (peaks 6, 7, and 12) varied with the state of the leaves (whether healthy, mined, or damaged).

Table 2 presents the identity of compounds, the diagnostic ions used as the basis for identification, and the quantities of the three main components. Most of the substances were oxygenated molecules and included primary and tertiary alcohols (1-pentanol, *cis*-3-hexen-1-ol, 3-octanol, 5-octa-5-dien-3-ol), aldehydes (2-butenal, propanal-2-methyl oxime, and 2,4-heptadienal), or ketones (4-hydroxy-4-methyl-2-pentanone, previously identified by Hefetz and Lloyd (1983) in a formicine (ant) anal gland. Some short-chain hydrocarbons were identified also, including *n*-dodecene (peak 3), *n*-tridecane (peak 5), 4,8-dimethyl tetradecane (peak 14), and the monoterpene camphene (peak 2). Table 2 and Figures 7 and 8 show that the volatile profiles of healthy, mined, and damaged plants did not differ qualitatively with the exception of 1-octen-3-ol. This compound was present only in mined leaves at the same retention time as that of 2,4-heptadienal.

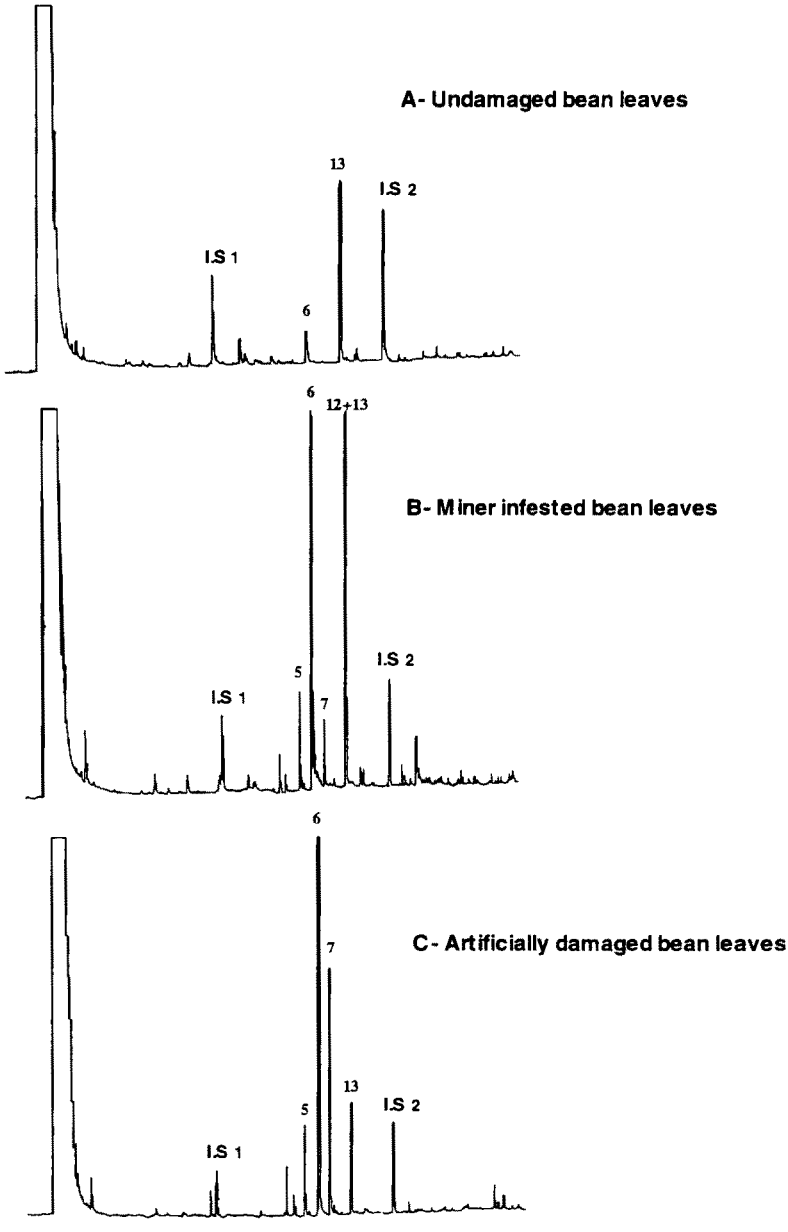

FIG. 7. Gas chromatogram (Supelcowax 10) of volatiles from undamaged bean leaves (A), damaged bean leaves containing feeding *Liriomyza trifolli* larvae (B), and artificially damaged bean leaves (C). Internal standards (IS1: *n*-docosane and IS2: octanol) are shown.

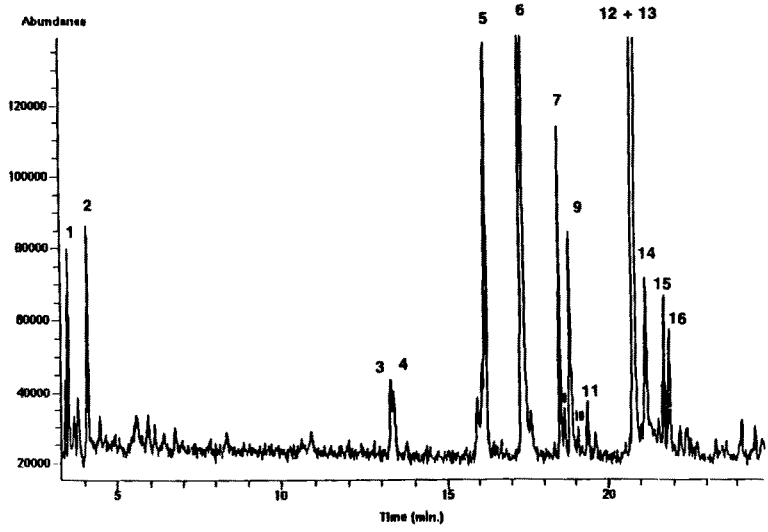

FIG. 8. Total ion chromatogram of damaged beans with numbered peaks: (1) 2-butenal, (2) camphene, (3) *n*-dodecene, (4) 1-pentanol, (5) *n*-tridecane, (6) 4-hydroxy-4-methyl-2-pentanone, (7) *cis*-3-hexen-1-ol, (8) unknown, (9) propanal, 2-methyl oxime, (10) 3-octanol, (11) unknown, (12) 1-octen-3-ol, (13) 2,4-heptadienal, (14) 4,8-dimethyl tetradecane, (15) 5-octa-1,5-dien-3-ol, (16) unknown.

Determinations were conducted on three of the main substances in a dozen samples of soaked healthy, mined, and damaged leaves. The amounts of all three substances increased whenever the leaves had been either attacked by biological agents or by the activity of the larvae or artificially damaged. Alcohols such as *cis*-3-hexen-1-ol (peak 7), which could not be quantified in the healthy leaves because it was present at such low levels, increased to as much as 600 ng/g of leaf matter in mined leaves and up to 700 ng/g in artificially damaged leaves. Likewise, 1-octen-3-ol (peak 12), which was not detected in the healthy leaves, reached the level of 1200 ng/g in mined leaves. 1-Octen-3-ol was not detected in extracts from damaged leaves. Lastly, 4-hydroxy-4-methyl-2-pentanone (peak 6) was extracted from the healthy leaves at about 350 ng/g, but it increased to as much as 6800 ng/g in mined leaves and 5700 ng/g in damaged leaves.

DISCUSSION

D. isaea are attracted by odor signals emitted by plants and by the host insects living on these plants. The results of our experiments using cold-trapped

TABLE 2. IDENTIFICATION OF VOLATILES BASED ON GC-MS EI IONIZATION

Peak	Compound	Formula	Diagnostic EI-MS ions	MW	Undamaged	Damaged	Artificially damaged
1	2-Butenal	C_4H_6O	39, 41, 56, 70 (M^+)	70	+	+	+
2	Camphene	$C_{10}H_{16}$	79, 93, 121, 136 (M^+)	136	+	+	+
3	Dodecene	$C_{12}H_{24}$		168	+	+	+
4	1-Pentanol	$C_5H_{12}O$	42, 55, 70	88	+	+	+
5	*n*-Tridecane	$C_{13}H_{28}$	43, 57, 71, 85, 184 (M^+)	184	+	+	+
6	4-Hydroxy-4-methyl 2-pentanone	$C_6H_{12}O_2$	43, 59, 101 (M-15)$^+$	116	+ 350 ng/g	+ 6800 ng/g	+ 5700 ng/g
7	*cis*-3-Hexen-1 ol	$C_6H_{12}O$	41, 55, 67, 82 (M-18)$^+$	100	trace	+ 600 ng/g	+ 700 ng/g
8	Unknown						
9	Propanal 2-methyl oxyme	C_4H_9NO	41/2, 55, 70, 86, 87	87	trace	+	+
10	3-Octanol	$C_8H_{16}O$	41, 59, 83, 101, 112 (M-18)$^+$	130	trace	+	+
11	Unknown				+	trace	trace
12	1-Octen-3ol	$C_8H_{14}O$	43, 57, 72, 81, 85 99, 110 (M-18)$^+$	128	–	+ 1200 ng/g	–
13	2,4-Heptadienal	$C_7H_{10}O$	53, 81, 95, 110 (M^+)	110	+	trace	+
14	4,8-Dimethyl tetradecane	$C_{16}H_{34}$	70/1, 112/3, 140/1, 182/3, 211 (M-15)$^+$	226	trace	+	trace
15	5-Octa-1,5-dien-3-ol	$C_8H_{12}O$	41, 57, 70, 79, 93, 106 (M-18)$^+$	124	+	+	+

bean plant extracts to attract the parasitoids show that they are able to recognize bean plants on the basis of volatile chemical signals that the plants give off. With the cold-trapping technique used in these experiments, natural conditions are better reconstituted by eliminating heavy, less volatile compounds such as triterpenes and phenols that are removed during the soaking or crushing processes (Wolfender et al., 1993).

Bean plants infested with leafminer larvae release much higher quantities of volatile molecules from the leaves than do healthy, non-mined plants. Larvae feeding on these plants may be responsible for the enhanced production of these substances. In our experiments, *D. isaea* females placed in an olfactometer responded to these chemical signals by moving towards the odor source, and it seem likely that they behave in the same way in natural surroundings. When plants are attacked, they seem to release substances that guide the parasitoids to their hosts.

The molecules we identified here have been previously mentioned in the literature. *cis*-3-Hexen-1-ol, for instance, occurs in more than 25 plant families (Visser et al., 1979) and has been reported from healthy leaves of potato plants (Visser et al., 1979), cowpeas (Whitman and Eller, 1990), several crucifers (Wallbank and Wheatley, 1976), and in strawberry plant leaves (Buttery et al., 1984; Hamilton-Kemp et al., 1988). Saijo and Takeo (1975) established that *cis*-3-hexen-1-ol is produced by green plants when the leaf tissues are damaged.

1-Octen-3-ol has previously been reported from strawberry foliage (Hamilton-Kemp et al., 1988), in the leaves of a shrub endemic to Florida (Jordan et al., 1992), and in corn tassels (Buttery and Ling, 1980). In the present study, this compound was emitted only by mined leaves. The presence of 2,4-heptadienal was observed in both healthy and damaged leaves. It has also been detected in corn (Buttery et al., 1978; Buttery and Ling, 1980).

We also identified some quite rare substances, such as oximes, in the trapped volatiles obtained from mined and artificially damaged bean plant leaves. Oximes have been previously identified in apple leaves attacked by *Tetranichus urticae* (Takabayashi et al., 1991) and in cucumber leaves (Takabayashi et al., 1994).

D. isaea do not use only one pest species as a host, and the question arises as to whether there may exist a common set of molecules used by the parasitoid to detect the mined leaves of any of their potential hosts. In our studies the parasitoid seems to recognize volatiles common to several plants. Guerin et al. (1983) suggested that the recognition of a plant's odor may be based on a synergy between molecules that are common to a whole set of plants, plus some more specific molecules. If true, this hypothesis would explain why *D. isaea* are so effective in attacking several leafminer pests.

Although *D. isaea* responded actively to plant extracts used in our study, it should be remembered that these results were obtained under experimental

conditions. Each odor was presented separately in the olfactometer, whereas in natural surroundings, olfactory stimuli are more complex. In our study the volatile chemical signals emitted by the *D. isaea–L. trifolii–Phaseolus* complex mediated communications between the plants, the plant-eating insects, and the insect-eaters.

Green plants such as tomatoes, potatoes, and bean plants release volatile compounds that give cut leaves a characteristic "green" odor. These volatiles are mainly alcohols with six carbon atoms, such as *cis*-3-hexen-1-ol, *cis*-2-hexen-1-ol, 1-hexanol, and aldehydes such as *trans*-2-hexenal and their derivatives (Visser et al., 1979; Hamilton-Kemp et al., 1988, 1989; Hernandez et al., 1989).

In the present study, the proportions of two of the molecules released by bean plant leaves, *cis*-3-hexen-1-ol and 4-hydroxy-4-methyl-2-pentanone, increased considerably in plant leaves attacked by the agromyzid larvae and in those that had been mechanically lacerated. The release of these compounds, or possibly just one of them, may inform parasitoids that they are in the vicinity of damaged plants, and the plants subsequently benefit from the messages it has sent out.

In the chemical interactions described here, communications seem to occur between the first and third trophic levels. The emitter and the receiver of the chemical messages both benefited from the exchange (Whittaker and Feeny, 1971). Here the term "allomones" does not apply in the strictest sense since the interactions were reciprocal. Nordlund and Lewis (1976) proposed the term synomone, which seems to describe more appropriately the type of signals occurring in our model. Several authors (Whitman, 1988; Dicke and Sabelis, 1988, 1989; Dicke et al., 1990) have explained communication of this kind in terms of the plants calling for help when they are attacked by larvae. Elzen et al. (1983), Nadel and Van Alphen (1987), Dicke and Sabelis (1988), and Turlings et al. (1991) have established that herbivore-damaged plants are more attractive to parasitoids than healthy ones. The idea that plants may have developed a defense mechanism that elicits participation of insect parasitoids seems quite plausible. This hypothesis presupposes that there exists a long-standing association between the plants, the phytophagous insects, and the parasitoids.

Plants may be induced to emit attractants utilized by insect parasitoids by either the host saliva (Turlings et al., 1990), its excretory droppings (Ramachandran et al., 1991), and/or its cocoon (Bekkaoui and Thibout, 1993). In our experiments with the olfactometer, *D. isaea* showed some sensitivity to feces of leafminer larvae feeding on bean plant leaves. We were not able to extract the substance responsible for this attraction.

In locating a host, *D. isaea* wasps have to search for various potential hosts living on different plants growing in diverse habitats. In such a complex chemical

environment, simply searching for a single kairomonal chemical may not suffice for the parasitoid to be able to make the most of all the available resources.

Long-range signals probably will convey only information about the host's habitat, while more short-range semiochemical substances emanating from infested plants or from the host's feces or other secretions will provide more direct and more reliable information about the availability of hosts and the sites where they are to be found. A parasitoid searching for a host probably uses a continuous, dynamic flow of information to locate hosts. Some parasitoids appear to be able to make use of what they have previously experienced and learned in order to take advantage of this complex system of interactions (Vinson et al., 1977; Vet and Groenwold, 1990).

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RELATIONSHIPS BETWEEN *Dendroctonus micans* KUG. (COLEOPTERA: SCOLYTIDAE) SURVIVAL AND DEVELOPMENT AND BIOCHEMICAL CHANGES IN NORWAY SPRUCE, *Picea abies* (L.) KARST., PHLOEM CAUSED BY MECHANICAL WOUNDING

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Abstract—The bark of Norway spruce trees, *Picea abies* (L.) Karst., was wounded to produce areas of bark with differing biochemical characteristics. Adults and larvae of *Dendroctonus micans* Kug. were inserted into these trees at points around the wounds. Larval survival was higher, larval dry weights were higher, and adults were more likely to oviposit at sites around the wound that had higher phloem moisture contents. Larvae showed higher survival and dry weights in phloem with low terpene contents. Adults oviposited in phloem with lower stilbene glucoside contents. Phloem moisture differed at sites around the wound compared with that at a control site away from the wound, and the nitrogen content of entire bark cores was higher above wounds than at control sites away from the wounds. Phloem moisture correlated with the nitrogen content of entire bark. Phloem terpene contents correlated with each other, as did the stilbene contents. Associations between the host exploitation pattern of *D. micans* and the biochemistry of the host phloem are discussed in relation to preselection by adults of sites suitable for larval development.

Key Words—Norway spruce, *Picea abies*, *Dendroctonus micans*, tree defenses, terpenes, stilbene glucosides, phloem biochemistry, host exploitation.

INTRODUCTION

The susceptibility of living host trees to bark beetles has been shown to depend on factors that affect the whole tree, as well as localized areas on those trees.

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Tree stress has been artificially induced in many studies (e.g., Vité, 1961; Moeck et al., 1981; Coulson et al., 1986; Miller and Berryman, 1986). Freezing of the tree base of ponderosa pine, *Pinus ponderosa* Laws., caused severe moisture stress, which predisposed them to attack by bark and ambrosia beetles, including *Dendroctonus valens* LeConte, *D. ponderosae* Hopk., and *D. brevicomis* LeConte (Moeck et al., 1981). When loblolly pines, *P. taeda* L. were damaged by artificial lightning strikes, *D. frontalis* Zimm., *D. terebrans* (Oliv.), and several *Ips* spp. were frequently associated with the mortality of the trees (Coulson et al., 1986). Alleviation of tree stress, for example, by reducing the canopy density and improving the nutrition of the trees, has been used to demonstrate reduced predisposition to bark beetle attack (Waring and Pitman, 1983, 1985). Lodgepole pines, *P. contorta* Dougl. significantly increased their resistance to mountain pine beetle, *D. ponderosae*, when canopy density was decreased and nitrogen nutrition improved (Waring and Pitman, 1983).

More localized areas within trees have also been investigated. For example, *D. ponderosae* favored sites around mechanical wounds on *P. contorta* that had a high carbohydrate content (Miller and Berryman, 1986). The development of late instars of *D. frontalis* was slower on bark with a high moisture content, but early instars developed more slowly in bark with a much decreased phloem moisture content (Wagner et al., 1979). Overall brood survival of *D. frontalis* was lower in trees with a high phloem moisture content (Webb and Franklin, 1978).

The defensive compounds found in trees are important in tree resistance to bark beetles. Monoterpene vapors vary in their toxicity to *D. brevicomis*. For example, limonene vapor, an important constituent of the resinous defenses of *P. ponderosa*, caused high levels of mortality to adult *D. brevicomis* (Smith, 1965). Contact of the fir engraver, *Scolytus ventralis* LeConte, with cortical resin blisters or canals in white fir, *Abies concolor* (Gord. & Glend.) Lindl. ex Hildebr., resulted in failure to produce egg galleries (Ferrell, 1983). Other defensive compounds found in conifers, such as the stilbene glucosides, which act as antifungal substances in spruce trees (Woodward and Pearce, 1988), may have activity against insects.

The great spruce bark beetle, *D. micans* Kug., is a pest of spruce, *Picea* spp., and other conifers in Europe (Grégoire, 1988). In the United Kingdom, infestations of this insect occur most frequently on Norway spruce, *Picea abies* (L.) Karst., and Sitka spruce, *P. sitchensis* (Bong.) Carr. (King and Fielding, 1989). Outbreaks of *D. micans* have been positively correlated to periods of prolonged drought and negatively correlated to stand density (Shavliashvili and Zharkov, 1985). Due to considerable variation in these types of observations, opposite effects have been demonstrated in other studies. For example, Granet and Perrot (1977) observed a positive relationship between attack and stand density.

In contrast with many other bark beetle species, *D. micans* larvae feed *en masse* throughout their development. Mass attack of trees by adults does not occur, and mating among siblings occurs in their brood chamber. No association with plant pathogenic fungi has been demonstrated (King and Fielding, 1989; Grégoire, 1984; Lieutier, 1993). Adults initiate attack on trees that appear to be healthy, and host defenses are tolerated or overcome on a localized basis. The success of attack is not related to the induced defense reaction of the host (Lieutier et al., 1992). Within a spruce stand, some trees may not be attacked despite multiple *D. micans* attacks on neighboring trees (Bevan and King, 1983). In addition, individual trees may have a number of aborted attacks on them, as well as some successful attacks (Bevan and King, 1983). These patterns of attack may be a result of between- and within-tree variation in the suitability of hosts for successful attack by *D. micans*. Physical and biochemical differences in host trees may be important in explaining these phenomena.

The experiments reported here aimed to produce a range of chemical and physical bark characteristics by mechanical wounding of trees. The null hypotheses tested were that variation in bark characteristics between trees, or as a result of tree wounding, would have no effect on oviposition by *D. micans* and/or the survival and development of *D. micans* larvae.

METHODS AND MATERIALS

Tree Wounding. Bark strips, 5 mm wide, were removed from trees to produce a standard pattern of wounding modified from the patterns used by Miller and Berryman (1986) (Figure 1). The phloem was completely severed along these strips by using a razorblade knife. The six sites at which beetles were inserted around the wound varied in the degree to which vertical and horizontal movement of materials into or away from the site was possible through the phloem. Wounds of this pattern were made on trees that had a minimum diameter at breast height (DBH) of 18 cm three weeks prior to the insertion of beetles. Wounds were made on an area of bark away from branch nodes, and at 1.7 m above the ground. It was hypothesized that the biochemical characteristics and moisture content of the phloem would vary around the wound site and that these could be correlated to oviposition by *D. micans* and the development and survival of larvae. Site 2 was on the opposite side of the tree to the wound, and was regarded as a control site for comparisons with other locations around the wound.

Insertion of Larval D. micans *into Wounded Trees.* Ten trees were selected from a pool of Norway spruce trees that were wounded on June 30, 1988, at Frostle Wood, Shropshire, England. Second and third instars of *D. micans* reared in the laboratory on Norway spruce logs were allocated at random to 60

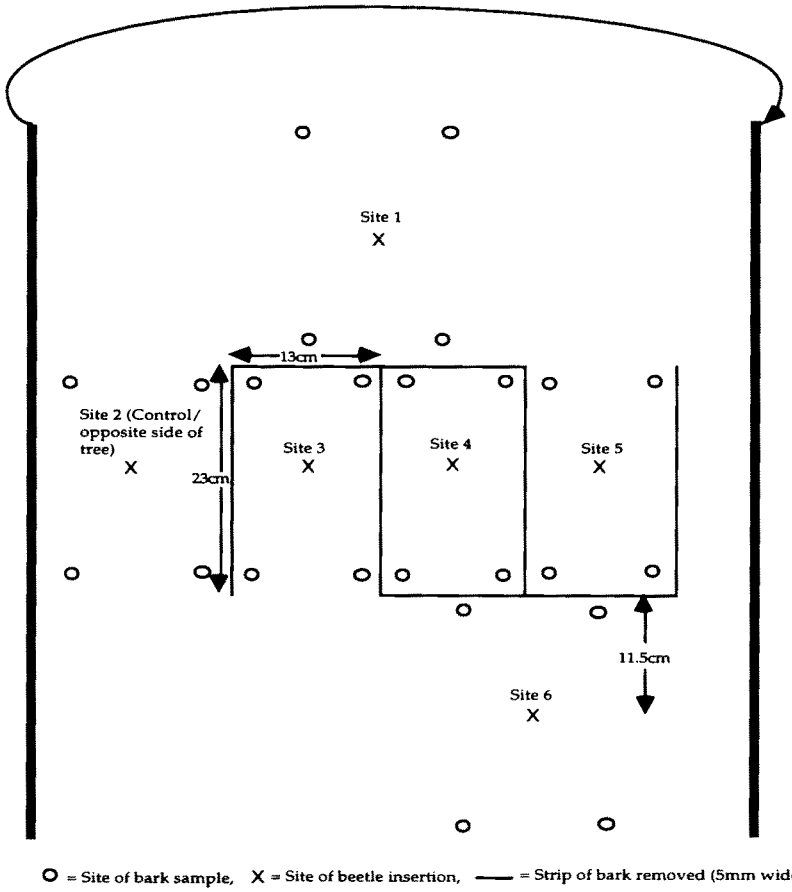

FIG. 1. The standard wounding pattern used in experiments to manipulate biochemical characteristics and moisture content of phloem on Norway spruce trees.

broods of 10 larvae each. After determining the mean fresh weight of the larvae in each brood, six broods were inserted into each of the wounded trees three weeks after the wounding had been carried out. An inverted v-shaped cut was made into the bark at the insertion point, and the living phloem was removed to leave a triangular flap of dead outer bark. The larvae were carefully placed (to prevent crushing) behind the flap of bark and secured by stapling brown wax paper over the insertion point.

Two bark samples, selected at random from the four bark sampling sites

around each of the six insertion points (Figure 1), were taken two weeks after insertion of the larvae. These samples were used to assess the percentage moisture, and the terpene content of the phloem. Two weeks after bark sampling, all larval broods were excavated from the trees, and the larvae were placed in compartmentalized boxes at 4°C for two days to empty their guts. They were then dried in an oven, and weighed to determine their dry weight.

Correlation coefficients were calculated to investigate the relationships between survival and mean dry weight of larvae at the end of the experiment and the biochemical characteristics of the phloem (Sokal and Rohlf, 1981). The dependence of larval survival on the position of the brood in relation to the wound was determined with χ^2 tests (Zar, 1984), and differences in larval dry weight at the sites around the wounds were tested by analysis of variance (Sokal and Rohlf, 1981). In the χ^2 tests and analysis of variance, five contrasts were carried out between the control site (site 2) and each of the other sites around the wound (Zar, 1984).

Insertion of Adult D. micans *into Wounded Trees.* Individual adults of *D. micans* were inserted at the six sites around the wound on each of 10 trees selected at random from the pool of trees wounded on June 30, 1988. The beetles were selected from a population of 100 brood adults that were fully tanned and assumed to be ready to emerge and attack host trees. They were collected from an infested tree at Wyre forest, Wales, and were assumed to be female based on their large size (Robinson et al., 1984). Determination of sex by morphological characteristics (Robinson et al., 1984) was not carried out due to the risk of injury to the limited number of beetles available. Insertions were made three weeks after wounding by removing a 9.8-mm-diameter bark and phloem disk from the insertion point, placing the beetle in the hole produced in the bark, and confining it with a plastic cap secured with a pin.

Three bark samples, selected at random from the four bark sampling sites around each of the six insertion points (Figure 1), were taken two weeks after insertion of the adults. These were used to determine the percentage moisture, terpene and stilbene glucoside content of the phloem, and the percent nitrogen content of the bark. The terpene and stilbene glucoside contents of the phloem were assessed for five of the 10 trees, and the other characteristics were assessed for all of the trees. Four weeks after beetle insertion, galleries were excavated. The presence of a brood chamber and eggs was recorded as a successful insertion (Lieutier et al., 1992). In all cases where oviposition had not occurred, the adult had exited the tree.

A t test was used to determine differences in bark characteristics between sites where insertions were successful and where they were not. The dependence of oviposition on the site around the wound was determined by a χ^2 test. Separate χ^2 tests compared the control site with each of the other sites (Zar, 1984).

Assessment of Bark Characteristics. Bark samples were collected from beyond the limit of any visible darkening of the phloem in response to the wounding (up to 2 cm from the cut edge of the bark), by removing a core of bark with a cork borer (9.8 mm ID). With the exception of the bark cores for nitrogen determination, the outer bark was removed from the phloem tissue.

Percentage Moisture Content. Phloem cores were placed in air-tight plastic vials of known weight in a cool box, and weighed to determine fresh weight. Phloem cores were then dried in an oven at 80°C for 48 hr and reweighed. The percentage moisture content of the phloem was then calculated. The term moisture content was preferred over water content, because during the drying process volatiles in the phloem, such as terpenes, as well as water were evaporated.

Terpene Content. Gas chromatography (GC) was used to detect and quantify terpenes in the phloem core (von Rudloff, 1969). Each phloem core was placed in a glass screw-top vial with 3 ml of methanol. The weight of the vial and methanol was known, and reweighing with the phloem core permitted determination of the phloem fresh weight. The samples were placed on Dry Ice in the field, and then stored at −20°C. After three days the methanol was poured into another glass vial and replaced with a fresh 3-ml wash of methanol. After three more days the two wash samples were pooled, producing 6 ml of phloem extract. All phloem washes were kept at −20°C during the production and storage of the phloem extracts. Since the phloem cores were small (9.8 mm diameter), they were not cut up to improve extraction.

Samples (2 μl) were analyzed with a Carlo Erba Fractovap 4130 gas chromatograph equipped with an LT programmer and a Varian Series 8000 autosampler. An OV-1 fused silica capillary column (25 m × 0.32 mm ID, film thickness 0.5 μm, stationary phase polydimethylsiloxane) was used. Air and hydrogen flows to the flame ionization detector were 500 ml/min and 10 ml/min, respectively. The carrier gas was helium at a flow of 3 ml/min. The injector and detector temperatures were 200°C and 250°C, respectively. The column temperature was 50°C for the first 2 min, and then was increased at 4°C/min to a final temperature of 170°C. Separated chemical constituents were recorded as absolute amounts (microliters per milliliter) and identified by comparison with known pure terpene standards. The quantities were converted into microliters per gram of phloem fresh weight.

Stilbene Glucoside Content. The two major stilbene glucosides in spruce bark are astringin (5,3′,4′-trihydroxystilbene-3-β-D-glucoside) and isorhapontin (5,4′-dihydroxy-3′-methoxystilbene-3-β-D-glucoside), and techniques for their quantification have been developed (Woodward and Pearce, 1988).

Aliquots (20 μl) of the extract prepared for terpene determination were separated by reverse-phase high pressure liquid chromatography (HPLC (Novapak C-18 Radial-Pak cartridge column, 10 cm × 8 mm ID) by using a solvent system of water containing 10 mg/liter EDTA (solvent A), and methanol alcohol

(MeOH) (solvent B). An elution gradient was used with an initial solvent ratio of 3:1 (A:B), with a linear change to a ratio of 13:7 after 3 min, and finally changing to a ratio of 3:7 in a concave curve (Waters 600 gradient controller, curve 8) at 15 min. This increased linearly to 100% B at 17 min, and was maintained at this composition for a further 5 min before returning to the initial solvent composition (via 100% A) over a total cycle (including equilibration at the initial conditions) of 40 min. A flow rate of 1.5 ml/min was used, and detection was by UV absorbance at 325 nm. A C-18 precolumn cartridge (μ-Bond-a-pak) was used to protect the column. Stilbene glucosides were quantified on a peak area basis against an external, purified isorhapontin standard. Astringin was estimated by using a conversion factor based on the molar extinction coefficients of these two compounds, taken as 4.37 for astringin and 4.35 for isorhapontin (Toscano Underwood and Pearce, 1991). Stilbene contents were expressed as milligrams per gram of fresh phloem weight.

Nitrogen Content. Bark cores, with outer bark attached, were placed in plastic capsules of known weight on Dry Ice. After reweighing the capsules to establish the weight of the bark pieces, samples were stored at $-20°C$. The total nitrogen content of 50-mg bark samples was assessed by digestion of the organic nitrogen by the Kjeldahl procedure (Allen, 1974), and the resulting ammonia was measured by colorimetric techniques (Havilah et al., 1977). The absorbance of the ammonia–salicylate complex was read at 660 nm in a Technicon Autoanalyzer II. Results were calculated as percentage nitrogen by comparison with standard solutions.

Variation in Bark Characteristics Around Standard Pattern of Wounding. Analyses of variance were used to compare the bark characteristics around the standard wound. Five contrasts were carried out between the control site (site 2) and each of the other sites around the wound.

RESULTS

Effects of Bark Characteristics on Larval Development. The mean fresh weight of larvae at the start of the experiment did not correlate with the mean dry weight of larvae at the end of the experiment ($r = 0.00$, 51 *df*, $P > 0.05$) or with the number of larvae recovered at the end of the experiment ($r = 0.06$, 58 *df*, $P > 0.05$). Hence the outcome of the experiment was not related to the weights of the larvae at the start of the experiment. Correlation coefficients showed that larvae achieved greater mean final dry weights and had greater chances of survival in phloem with a high moisture content and low α-pinene, β-pinene and δ-3-carene content (Table 1). There was no significant correlation between levels of β-phellandrene and larval survival and final dry weight.

Effects of Bark Characteristics on Success of Adult Insertions. Higher adult

TABLE 1. CORRELATIONS AMONG BIOCHEMICAL CHARACTERISTICS OF PHLOEM, NUMBER OF SURVIVING *D. micans* LARVAE AND MEAN DRY WEIGHT OF SURVIVING LARVAE

		No. of larvae recovered (range 2–10)			Mean dry weight of larvae (mg) (range 0.95–8.41)		
Phloem characteristic	Range	*r*	*df*	*p*	*r*	*df*	*p*
Moisture content (%)	53.55–74.02	0.318	51	<0.05	0.537	51	<0.01
α-Pinene (μl/g)	0.067–1.044	−0.536	22	<0.01	−0.488	22	<0.05
β-Pinene (μl/g)	0.050–2.258	−0.568	22	<0.01	−0.470	22	<0.05
δ-3-Carene (μl/g)	0.057–0.966	−0.472	21	<0.05	−0.451	21	<0.05
β-Phellandrene (μl/g)	0.018–1.131	−0.230	21	>0.05	−0.353	21	>0.05

success rates occurred in the phloem at insertion sites with higher moisture contents ($P < 0.05$) and lower levels of astringin ($P < 0.05$) and total stilbenes ($P < 0.05$) (Table 2). There were no significant differences in the levels of isorhapontin, and therefore the significantly lower levels of total stilbenes at sites where oviposition occurred may be a reflection of the lower astringin levels at these sites.

Relationships Between Adult Success and Larval Development, and Standard Wounding Pattern. Adults were less likely to oviposit at sites 1, 3 and 5

TABLE 2. COMPARISON OF BIOCHEMICAL CHARACTERISTICS OF PHLOEM AT SITES WHERE OVIPOSITION BY *D. micans* DID AND DID NOT OCCUR

	Oviposition (mean ± SE)[a]	
Phloem characteristics	Yes	No
Moisture content of phloem (%)	62.83a ± 0.778	60.20b ± 0.565
Astringin content of phloem (mg/g)	26.53a ± 1.514	32.76b ± 2.653
Isorhapontin content of phloem (mg/g)	8.12a ± 0.647	10.53a ± 1.315
Total stilbene content (mg/g)	34.64a ± 1.890	43.29b ± 3.642
α-Pinene content of phloem (μl/g)	0.29a ± 0.081	0.28a ± 0.052
β-Pinene content of phloem (μl/g)	0.45a ± 0.154	0.45a ± 0.092
δ-3-Carene content of phloem (μl/g)	0.16a ± 0.035	0.30a ± 0.123
β-Phellandrene content of phloem (μl/g)	0.22a ± 0.056	0.19a ± 0.035
Total nitrogen content of bark (%)	0.55a ± 0.035	0.57a ± 0.028

[a] Values in same row followed by different letters are significantly different (*t* test, $P < 0.05$).

TABLE 3. RELATIONSHIPS AMONG OVIPOSITION BY ADULT *D. micans*, SURVIVAL, MEAN DRY WEIGHT OF *D. micans* LARVAE, AND STANDARD WOUNDING PATTERN[a]

	Site					
	1	2	3	4	5	6
Adults						
Proportion laying eggs (N = 10)	0.3	0.8	0.1	0.6	0.2	0.5
Probability of difference from control (site 2)	*		**	NS	**	NS
Larvae						
Proportion surviving (N = 100)	0.66	0.53	0.48	0.48	0.49	0.6
Probability of difference from control (site 2)	NS		NS	NS	NS	NS
Mean final dry weight (mg)	2.88	2.43	2.05	3.79	2.06	3.05
Standard error	0.42	0.44	0.42	0.42	0.44	0.40
Probability of difference from control (site 2)	NS		NS	**	NS	*

[a] Probabilities are from χ^2 tests and analysis of variance. NS = $P > 0.05$, * = $P < 0.05$, and ** = $P < 0.01$. In each case, differences between the trees in the experiment are significant ($P < 0.01$). Standard errors of mean final dry weights of larvae are calculated from the residual mean square in the analysis (Zar, 1984).

than at the control, but were as likely to oviposit at sites 4 and 6 ($P < 0.05$) (Table 3). There was no difference in larval survival at the insertion points close to the wounds (sites 1, 3, 4, 5, and 6) compared with the control (site 2), but mean larval dry weights were higher at sites 4 and 6 than at the control ($P < 0.05$), and no different from the control at sites 1,3 and 5. In each case, variation between trees was significant (adults: $\chi^2 = 14.0$, 9 *df*, $P < 0.01$; larval survival: $\chi^2 = 62.2$, 9 *df*, $P < 0.01$; mean larval dry weight: $F = 4.01$, 9, 39 *df*, $P < 0.01$).

Variation of Bark Characteristics Around Standard Wounding Pattern. Phloem moisture contents were lower at sites 1, 3, 5 ($P < 0.01$) and 6 ($P < 0.05$) than at control site 2, and higher at site 4 ($P < 0.05$) (Table 4). Total nitrogen content of the bark was significantly higher at site 1 than at control site 2 ($P < 0.05$), but there were no significant differences between other sites and control site 2 (Table 4). All of the bark parameters considered differed between trees in the study ($P < 0.05$).

Relationships Between Bark Characteristics. Isorhapontin levels correlated positively with the levels of astringin, and the levels of each of these stilbenes correlated negatively with the moisture content of the phloem (Table 5). Four of the six correlations between the terpenes were significantly positive, and

TABLE 4. ANALYSIS OF PHLOEM CHARACTERISTICS AROUND THE STANDARD WOUNDING PATTERN

Mean of Each Bark Characteristic at Each Site ± S.E.

Phloem characteristic	Site 1	2	3	4	5	6
Moisture (%)	61.24 ± 0.33**	62.55 ± 0.33**	60.90 ± 0.33**	64.24 ± 0.33**	61.01 ± 0.33**	61.60 ± 0.33*
Astringin (mg/g)	31.56 ± 2.80	29.45 ± 2.80	29.72 ± 2.80	28.83 ± 2.80	26.82 ± 2.80	30.24 ± 2.80
Isorhapontin (mg/g)	9.61 ± 1.08	8.71 ± 1.08	7.91 ± 1.08	8.85 ± 1.08	9.14 ± 1.08	11.28 ± 1.08
Total stilbene (mg/g)	41.17 ± 3.59	38.15 ± 3.59	37.63 ± 3.59	37.69 ± 3.59	35.96 ± 3.59	41.52 ± 3.59
α-Pinene (μl/g)	0.32 ± 0.07	0.23 ± 0.08	0.26 ± 0.06	0.36 ± 0.08	0.34 ± 0.07	0.41 ± 0.07
β-Pinene (μl/g)	0.63 ± 0.14	0.43 ± 0.16	0.44 ± 0.14	0.70 ± 0.18	0.64 ± 0.15	0.77 ± 0.15
δ-3-Carene (μl/g)	0.40 ± 0.08	0.11 ± 0.09	0.25 ± 0.08	0.17 ± 0.10	0.29 ± 0.09	0.16 ± 0.09
β-Phellandrene (μl/g)	0.32 ± 0.06	0.26 ± 0.07	0.15 ± 0.06	0.25 ± 0.08	0.26 ± 0.07	0.29 ± 0.07
Total nitrogen (%)	0.63 ± 0.04*	0.50 ± 0.04	0.56 ± 0.04	0.53 ± 0.04	0.57 ± 0.04	0.56 ± 0.04

Standard errors calculated from the residual mean squares in the analyses (Zar, 1984).
*Differs from site 2 (analysis of variance, $P < 0.05$).
**Differs from site 2 (analysis of variance, $P < 0.01$).
Other comparisons with site 2 are not significant.

TABLE 5. CORRELATIONS OF PHLOEM CHARACTERISTICS OF NORWAY SPRUCE FOLLOWING MECHANICAL WOUNDING AND INSECT ACTIVITY

		α-Pinene	β-Pinene	δ-3-Carene	β-Phellandrene	Moisture (%)	Nitrogen (%)	Astringin
Isorhapontin (mg/g)	*r*	−0.065	−0.037	−0.128	−0.144	−0.380	0.017	0.635
(range 3.88–18.98)	*df*	22	22	22	22	28	28	28
	P	>0.05	>0.05	>0.05	>0.05	<0.05*	>0.05	<0.01**
Astringin (mg/g)	*r*	−0.101	−0.014	−0.231	−0.055	−0.541	−0.180	
(range 14.56–47.03)	*df*	22	22	22	22	28	28	
	P	>0.05	>0.05	>0.05	>0.05	<0.01**	>0.05	
Nitrogen (%)	*r*	0.088	0.014	0.320	0.024	0.373		
(range 0.36–1.32)	*df*	22	22	22	22	58		
	P	>0.05	>0.05	>0.05	>0.05	<0.01**		
Moisture (%)	*r*	0.087	0.081	0.042	0.251			
(range 53.55–74.02)	*df*	48	48	48	48			
	P	>0.05	>0.05	>0.05	>0.05			
β-Phellandrene (μl/g)	*r*	0.661	0.926	0.141				
(range 0.00–1.21)	*df*	48	48	48				
ρ values	*P*	<0.01**	<0.01**	>0.05				
δ-3-Carene (μl/g)	*r*	0.618	0.273					
(range 0.05–1.23)	*df*	48	48					
	P	<0.01**	>0.05					
β-Pinene (μl/g)	*r*	0.820						
(range 0.05–2.26)	*f*	48						
	P	<0.01**						
α-Pinene (μl/g)								
(range 0.05–1.16)								

*$P < 0.05$, **$P < 0.01$.

nitrogen content of the whole bark correlated positively with the moisture content of the phloem.

DISCUSSION

The response of trees to wounding was variable, but variation in bark characteristics between the trees was significant in all cases. Therefore, while larvae or adults may be more successful at sites with certain phloem characteristics, the effect of tree wounding may not produce consistent variations in bark characteristics in relation to the wound. As a result, certain sites around the wound on one tree may be suitable for *D. micans*, but a corresponding site on a different tree may be unsuitable.

Larval survival and mean dry weight were inversely correlated with the levels of three monoterpenes (α-pinene, β-pinene and δ-3-carene) in the phloem and positively correlated with phloem moisture content. Larval survival and mean dry weight differed among the trees in the study. No consistent differences in terpene contents were evident at the different sites around the wounds, but phloem moisture content was higher at site 4 than the control and lower at the other sites than the control. The higher moisture content of the phloem at site 4 may therefore explain the higher larval weights attained at these sites. Although site 6 had a significantly lower moisture content in the phloem than the control site, larvae still attained greater mean final dry weights than at control site 2. It appears that other factors may be important at site 6 or that the other bark characteristics that were assessed were too variable to produce significant differences between site 6 and the control.

Adult *D. micans* were more likely to oviposit at sites with higher moisture contents and lower astringin and total stilbene contents in the phloem. Oviposition was less likely to occur at sites 1, 3, and 5 than at the control site, and each of these sites had lower phloem moisture contents than the control. Site 6 also had a lower phloem moisture content than the control, and site 4 had a higher phloem moisture content than control site 2; adults were as likely to oviposit at these sites as they were at the control site. Oviposition varied among trees, as did all of the phloem characteristics assessed.

The sites at which larvae achieved greater mean dry weights (sites 4 and 6) corresponded with the sites where adults were as likely to oviposit as they were at the control site. Conversely, at sites 3 and 5, larvae did not attain greater mean final weights than at control site 2, and oviposition was lower at these two sites than at the control site. Hence, adults appear to select oviposition sites suitable for larval development.

Moisture and nitrogen content were positively correlated, as they are in other plant tissues, and both are important in insect nutrition (Scriber and Slan-

sky, 1981). Oviposition and larval weight were higher in phloem with a higher moisture content. However, in *D. frontalis* lower phloem moisture favored larval survival and development (Webb and Franklin 1978), up to a point where excessive phloem drying had occurred (Wagner et al., 1979). This result may reflect the suitability of phloem conditions for the growth of microorganisms (Franklin, 1970; Barras, 1970, 1973). No plant pathogenic fungi appear to be associated with *D. micans* (Lieutier, 1993), and hence larvae are not more successful at sites favoring fungal development. Since the nitrogen content of the bark was positively related to the percent moisture content of the phloem, larval success may be due to a higher nitrogen content in the bark (including the phloem), although nitrogen contents were not assessed in the experiment with larvae.

Higher terpene contents in the phloem resulted in reduced larval survival and reduced larval dry weight. Vapors of the major terpene components of spruce resin are not toxic to adult *D. micans*, and topical applications to larvae never resulted in greater than 30% mortality (Everaerts et al., 1988). However, the toxicity of terpene vapors to larvae and the contact toxicity of terpenes to adults are not known. Adult *D. micans* survive immersion in resin during host attack, so the contact toxicity of terpenes would appear to be low. The effect of resin crystallization rate on adults and larvae is also not known, although adults that have died in resin are rarely found in nature (Bevan and King, 1983).

Stilbenes are among the phenolic compounds implicated in antimicrobial biochemical defense in conifers. In spruce these compounds are present constitutively in the phloem as glucosides (Woodward and Pearce, 1988). Many bark beetle species have fungal associates (Whitney, 1982) and may therefore select trees with lower levels of stilbenes in the phloem. Adult *D. micans* were less likely to oviposit in phloem with higher astringin and total stilbene contents. Stilbene levels and moisture content of the bark were negatively correlated so it is impossible to determine whether the success of adult insertions was due to high moisture contents of the phloem or lower stilbene contents. Higher moisture contents favor larval development, but the effect of stilbene glucosides on larval development has not been considered. The presence of a fungal symbiont during the evolution of this species may be indicated by the fact that adult *D. micans* are less likely to oviposit in areas of bark with higher astringen and total stilbene contents. Furthermore, larvae may develop better in bark with lower stilbene glucoside levels, but further work is necessary to determine the effects of stilbene glucosides on *D. micans* larvae.

Host selection by *D. micans* may be determined in part by the suitability of the substrate for larval development. Hence, sites are favored with a higher moisture content in the phloem, which is positively correlated to the nitrogen content of the complete bark. If high quantities of monoterpenes are transported to the site, larval mortality may increase, with a subsequent reduction in larval weight. The packing of frass by adults around the eggs may act as a sponge for

the terpenes produced by the host tree. Hence, in broods resulting from natural adult attack, the importance of terpene content of the phloem to the larvae may be reduced. Adults may also select sites on trees with lower stilbene contents, even though fungi that would be expected to be sensitive to these compounds are apparently absent.

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GROWTH AND PHYSIOLOGICAL RESPONSES OF BLACK SPRUCE (*Picea mariana*) TO SITES DOMINATED BY *Ledum groenlandicum*

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Abstract—Growth and physiological parameters of black spruce planted eight years previously were compared at sites with and without *Ledum groenlandicum* to quantify the adverse effects on black spruce in *Ledum* sites. Tree height, crown diameter, and dry weights of stems, branches, and needles of black spruce were significantly less at the *Ledum*-dominated sites. Annual stem height, basal diameter, growth ring width, and cumulative wood volume of black spruce were significantly reduced in the *Ledum* sites compared to those of the non-*Ledum* sites. Yearly growth of black spruce in the first seven years after planting was more affected in the *Ledum* site than the eighth year's growth. Foliar nutrient analysis of black spruce in the *Ledum* site showed an overall imbalance in nutrient accumulation. Black spruce growing in the *Ledum* site had higher foliar concentrations of Al, Ca, Fe, Ni, and S and lower concentrations of N and Si than those growing in the non-*Ledum* site. Black spruce growing in the *Ledum* site also had a higher foliar phenolic content. Preliminary experiments suggest that *Ledum* has the potential to leach water-soluble phenolics to its immediate organic soil.

Key Words—*Ledum groenlandicum*, black spruce, nutrient imbalances, allelopathy, phenolics, growth check.

INTRODUCTION

Ledum groenlandicum (hereafter referred to as *Ledum*), commonly called Labrador tea, is a perennial ericaceous understorey shrub in boreal forests (Soper and Heimburger, 1985). Its distribution in Ontario ranges from Lake Ontario through Hudson Bay, and from Ottawa and the St. Lawrence Valley through Lake-of-the-Woods. *Ledum* is common in *Sphagnum* bogs and in moderately wet soils

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with thick humus layers. The plant regenerates well vegetatively following light fires that remove the aboveground component of the plant and the surface litter (Viereck, 1983).

LaBarron (1948) reported that *Ledum* could interfere with early growth of black spruce (*Picea mariana*). However, no data are available on growth and physiological damage of black spruce due to *Ledum*. Robert Bolton (personal communication) reported that approximately 100,000 ha of new plantations of black spruce of Abitibi-Price Limit, a paper factory, in the Iroquois Falls area alone experienced noticeable growth inhibition due to *Ledum*. Symptoms of black spruce growth inhibition in *Ledum*-dominated sites are similar to "growth check," a phenomenon observed in other conifers such as Sitka spruce (*Picea sitchensis*) inhibition due to *Calluna vulgaris* (Gimingham, 1972), western hemlock (*Thuja heterophylla*), red cedar (*T. plicata*) inhibition due to *Gaultheria shallon* (Weetman et al., 1990), and black spruce growth inhibition in the presence of *Kalmia angustifolia* (Mallik, 1994).

In boreal forests, black spruce is one of the most desirable tree species because of its superior fiber quality. Poor growth of planted black spruce in *Ledum* sites affects its overall productivity. Optimum plant growth is dependent upon a balance of nutrients in the leaves (Shear et al., 1948; Comerford and Fisher, 1984), and any imbalance in foliar nutrients may cause adverse effects on growth. In view of this, the growth and physiological responses of black spruce growing with and without *Ledum* were quantified. The objectives of the present study were to study the adverse effects on growth of black spruce growing in *Ledum*-dominated sites and to investigate if black spruce growing in *Ledum*-dominated sites have altered foliar chemistry.

METHODS AND MATERIALS

Study Site and Sampling. The study was conducted in a young black spruce stand of approximately 1 ha located beside Little Abitibi Road, Highway 67, in the Abitibi-Price forest area, Iroquois Falls (lat. 48°46'N; long. 80°41'). The area was planted with paper plug container-stock black spruce eight years previously. One half of this area, about 50 m south of the highway, was infested with *Ledum groenlandicum* with 50–80% cover and had a thick carpet of mosses (70–90% cover), mainly *Pleurozium schreberi, Dicranum* spp., and some *Sphagnum*. Other associated vascular plants were *Epilobium* spp., *Aster* spp., *Solidago* spp., *Viola* spp., *Carex* spp., *Vaccinium angustifolium*, and *V. myrtilloides*, each having less than 10% cover. The site had thick organic matter with undecomposed *Ledum* litter on top. The contiguous half of this area further away from the highway did not have *Ledum* as a component of the vegetation.

This site was relatively drier, with a thinner (ca. 30%) organic layer compared to the adjacent *Ledum* site. Mineral soil of the site was light-colored and clayey loam (for detailed soil classification, see Sims et al., 1989).

Ten planted black spruce trees were selected randomly from each site (with and without *Ledum*). The planted trees were identified by the presence of paper plugs at their stem bases. The trees were cut, numbered, and brought back to the laboratory for growth and foliar chemical analysis.

Growth Characteristics. Data on height and crown diameter of each tree from *Ledum* and non-*Ledum* sites were recorded. Oven-dry weights of stem, branches, and needles were recorded at 60°C until constant weight was achieved. All analyses were made in triplicate.

Chemical Analysis. Black spruce needles were analyzed for a number of nutrient: Al, As, B, Ba, Ca, Cd, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, P, S, Se, Sr, Ti, N, and Zn. Only needles were analyzed for elements, as leaf habit was suggested to be a major determinant of growth and nutrient use by forest species (Chapin and Tryon, 1983). Moreover, foliar nutrient analysis generally reflects soil nutrient conditions, even with different species compared at two sites (van den Driessche, 1970; Garten, 1976; Chapin and Tryon, 1983).

Needles from each of the trees from *Ledum* and non-*Ledum* sites were oven-dried at 70°C for 6 hr. After digestion with HNO_3, all elements except N were analyzed with an inductively coupled plasma analyzer (Jarrell-Ash ICAP 9000). Needle N was determined colorimetrically following Allen (1989). Percent ash was determined by burning the samples in a muffle furnace at 550°C for 5 hr.

To determine if the black spruce trees growing in the *Ledum* site were under some physiological stress and had high phenolic content, total foliar phenolic content was determined by Folin-Ciocalteau reagent following Swain and Hillis (1959).

Annual Growth. LaBarron (1948) reported that *Ledum* affected black spruce growth in its early phase of the life cycle and that eventually the tree may overcome the adverse effects of *Ledum*. Therefore, to determine whether early phases of black spruce growth were more sensitive to *Ledum*, year-wise data on shoot height and diameter of black spruce were collected. The width of each year's growth ring was determined by taking cross sections of stem at 0–10 cm above ground. The annual volume increase was calculated by the equation:

$$V = \frac{\pi D^2}{4} \times h \times 0.333 \qquad (1)$$

where V is the volume of the wood, D is the cumulative diameter, and h is the cumulative height of each tree.

Total Phenolic Content of Ledum*-Amended Organic Soils.* To determine if *Ledum* has the potential to leach water-soluble phenolics to the environment, organic soils previously not associated with *Ledum* were amended with *Ledum* leaves. Organic soil (15 g) was amended with 2.5, 5, and 10 g of air-dried *Ledum* leaves, and kept for 72 hr. These amended soils were identified as AS1, AS2, and AS3, respectively. Organic soils previously not associated with *Ledum* were used as controls. Total phenolic content of the control soil and amended soils (AS1–AS3) was determined following Swain and Hillis (1959).

RESULTS

Growth of black spruce trees was significantly inhibited in the *Ledum* site (Table 1). Tree height, crown diameter, and dry weights of stem, branches, and needles of black spruce were all significantly ($P < 0.001$) lower in the *Ledum* site compared to the non-*Ledum* site (Table 1). Annual growth of stem height and wood volume of black spruce were significantly ($P < 0.001$) higher in the non-*Ledum* site than the *Ledum* site (Figures 1 and 2). Growth of black spruce in the *Ledum* site was reduced for seven years following planting. However, in the eighth year a significant increase in stem height was observed compared to the previous years. Similarly, the difference in stem base diameter of black spruce between *Ledum* and non-*Ledum* sites was reduced in the eighth year. For example, compared to an 82% reduction in base diameter of black spruce in the *Ledum* site during the first year after planting, only a 38.6% reduction was recorded in the eighth year. Reduction in stem diameter of black spruce in the *Ledum* site relative to the non-*Ledum* site was 78%, 78%, 65%, 77%, 74%, and 66% in the second, third, fourth, fifth, sixth, and seventh years, respectively. Annual ring width of black spruce from the *Ledum* site was significantly

TABLE 1. BIOTIC CHARACTERISTICS OF 8-YEAR-OLD BLACK SPRUCE WITH AND WITHOUT *Ledum groenlandicum*[a]

Biotic characteristics	Non-*Ledum* site	*Ledum* site
Shoot height (cm)	196.77 ± 20.31	75.32 ± 16.12*
Crown diameter (cm)	72.18 ± 10.37	32.30 ± 14.44*
Dry weight of stem (g)	670.14 ± 294.55	24.23 ± 12.85*
Dry weight of branches (g)	532.89 ± 185.11	28.39 ± 15.83*
Dry weight of needles (g)	807.86 ± 212	42.77 ± 15.83*

[a]Data are average of ten tree samplings. The asterisk denotes that values are significantly different at the level of $P < 0.001$.

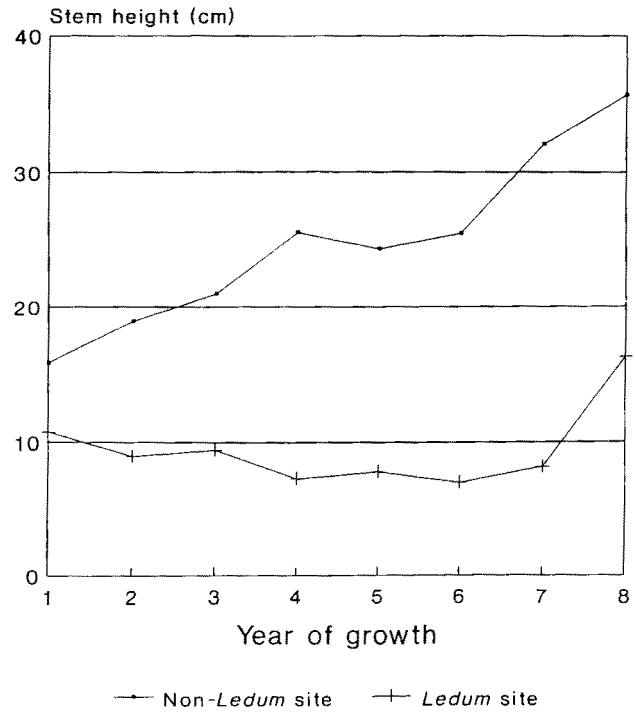

FIG. 1. Annual stem height of black spruce growing in non-*Ledum* and *Ledum* sites. Data are average of 10 tree samplings. Values are significantly different at the level of $P < 0.001$.

($P < 0.005$) less than that of the non-*Ledum* site (Table 2). There was a significant decline in volume of wood produced by black spruce growing in the *Ledum* site (Figure 2). Compared to the non-*Ledum* site, black spruce in the *Ledum* site suffered a 92.73% decline in volume of wood over the eight years.

Of the 21 nutrient elements analyzed, foliar concentrations of Al, Ca, Fe, Ni, N, and Si were significantly different. Concentrations of Al, S ($P < 0.005$), Ca, Fe, and Ni ($P < 0.05$) were significantly higher in needles of black spruce grown in the *Ledum* site. On the other hand, foliar N ($P < 0.05$) and Si ($P < 0.005$) concentrations were significantly lower in black spruce grown in the *Ledum* site compared to the non-*Ledum* site (Table 3). Needles of black spruce grown in the *Ledum* site also had higher total phenolic content compared to that of the non-*Ledum* site (Table 3).

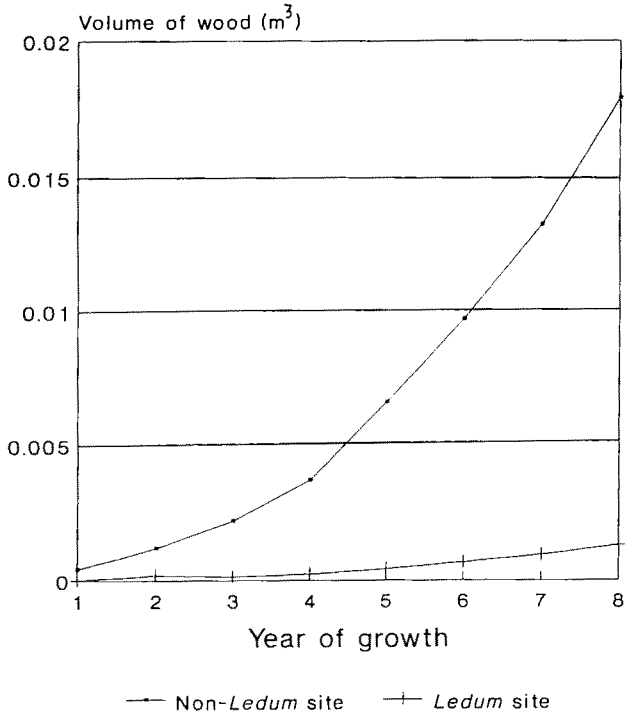

FIG. 2. Cumulative wood volume of black spruce growing in non-*Ledum* and *Ledum* sites. Data are average of 10 tree samplings. Values are significantly different at the level of $P < 0.001$.

Total phenolic content of soils amended with *Ledum* leaves (AS1–AS3) was significantly higher ($P < 0.005$) compared to unamended (control) soil (Table 4).

DISCUSSION

The present study unequivocally demonstrates a significant growth reduction and foliar nutrient imbalance in black spruce growing in the *Ledum* site compared to those growing in the non-*Ledum* site (Tables 1–3). Low foliar concentrations of N and Si and high foliar concentrations of Al, Ca, Fe, Ni, and S were observed in black spruce growing in the *Ledum* site compared to that of the non-*Ledum* site (Table 3). Optimum tree growth may be impaired due to nutrient imbalances (Comerford and Fisher, 1984). Reduced foliar N of

TABLE 2. ANNUAL WIDTH OF GROWTH RING OF BLACK SPRUCE TREES AT *Ledum* AND NON-*Ledum* SITE

	Annual width of growth ring (mm) since planting[a]							
Site	1	2	3	4	5	6	7	8
'ithout *Ledum*	0.3145 ± 0.0815	0.3971 ± 0.0968	0.4871 ± 0.1241	0.6764 ± 0.2202	0.5089 ± 0.1306	1.037 ± 0.3133	0.7676 ± 0.2309	0.4489 ± 0.1031
'ith *Ledum*	0.1092* ± 0.0548	0.1093* ± 0.0612	0.1027* ± 0.0571	0.1193* ± 0.0558	0.1297* ± 0.0721	0.1962* ± 0.0748	0.1686* ± 0.0516	0.1746* ± 0.0676

)ata are mean values of 10 trees ± SD. The asterisk denotes that values are significantly different at the evel of $P < 0.001$.

TABLE 3. PERCENT ASH, PERCENT NITROGEN, TOTAL PHENOLICS, AND ELEMENTAL ANALYSIS (ppm) OF FOLIAGE OF 8-YEAR-OLD BLACK SPRUCE TREES FROM *Ledum* AND NON-*Ledum* SITES[a]

Chemical characteristics	*Ledum* site	Non-*Ledum* site
Ash (%)	2.89 ± 0.66	2.35 ± 0.55
Al	0.4282 ± 0.1460	0.2690 ± 0.0608**
As	0.0163 ± 0.0083	0.0193 ± 0.0038
B	0.1380 ± 0.0409	0.1271 ± 0.0372
Ba	0.3256 ± 0.1171	0.4933 ± 0.2590
Ca	48.44 ± 10.01	37.12 ± 12.65*
Cd	0.0019 ± 0.0007	0.0020 ± 0.0009
Cu	0.0134 ± 0.0106	0.0410 ± 0.0130
Fe	0.5103 ± 0.1177	0.4027 ± 0.0594*
Hg	0.0115 ± 0.0042	0.0173 ± 0.0048
K	20.21 ± 4.35	18.17 ± 1.16
Mg	3.55 ± 0.468	3.89 ± 0.525
Mn	8.38 ± 2.69	9.68 ± 2.13
Na	0.3104 ± 0.852	0.6478 ± 0.257
Ni	0.0104 ± 0.0091	0.0068 ± 0.0024*
P	5.849 ± 0.7351	5.4253 ± 0.4002
S	3.58 ± 0.49	2.92 ± 0.29**
Se	0.0293 ± 0.0087	0.0352 ± 0.0085
Si	0.0533 ± 0.0286	0.1353 ± 0.0402**
Sr	0.0754 ± 0.0272	0.0577 ± 0.0265
Ti	0.0109 ± 0.0053	0.0086 ± 0.0029
Zn	0.5281 ± 0.1192	0.4280 ± 0.1135
N (%)	1.219 ± 0.511	1.818 ± 0.609*
Total phenolics ($mg/liter^{-1}$)	20.327 ± 2.25	17.65 ± 2.89*

[a] Data are average values of 10 tree samplings. Values are significantly different at the level of $*P < 0.01$ and $**P < 0.001$.

TABLE 4. TOTAL PHENOLIC CONTENT OF ORGANIC SOILS AMENDED WITH DIFFERENT CONCENTRATIONS OF *Ledum* LEAVES

Soil	Total phenolic content[a]
CS[b]	1.6×10^{-5}M
AS1[c]	4.98×10^{-5}M
AS2	1.06×10^{-4}M
AS3	1.23×10^{-4}M

[a] All values are significantly different from each other at the level of $P < 0.001$.
[b] Unamended organic soil.
[c] Organic soil amended with 2.5 g (AS1), 5.0 g (AS2), and 10 g (AS3) *Ledum* leaves.

black spruce growing in the *Ledum* site may be the cause of its poor growth because N deficiency can directly affect plant growth (Stone and Will, 1965). Munson and Timmer (1989a,b) suggested that seedling growth and productivity of conifers following outplanting depend on N uptake and accumulation. Interference with N accumulation and growth was evident from the results of this study, as black spruce of the non-*Ledum* site had better growth and a higher N concentration compared to those in the *Ledum* site (Tables 1 and 3). Lavender (1970) found lower foliar N in suppressed Douglas fir trees than in dominant trees. van Dijk and Roelofs (1988) reported that imbalances in N with K, Mg, and P were correlated with arginine accumulation. A lower P/N ratio was observed in black spruce growing in the non-*Ledum* site (2.984) than in trees growing in the *Ledum* site (4.798). A high P/N ratio could result in higher foliar concentrations of Al, Ca, Fe, Ni, and S in black spruce growing in the *Ledum* site (Chapin and Tryon, 1983; Ericsson et al., 1993; Penning de Varies, 1975). Various studies showed that, compared to dominant trees, nondominant trees had higher tissue nutrient concentrations (Wright and Will, 1958; Madgwick, 1964; van den Driessche, 1974). Although experimental evidence of this relationship in black spruce requires further studies, the present study showed that black spruce growing in the *Ledum* site suffers from nutrient imbalances, and that may be a reason for, or a result of, its poor growth.

Ca is an important nutrient in a forest ecosystem because of its large accumulations in forest trees (Alban et al., 1978). In the present study significantly higher concentrations of Ca were recorded in needles of black spruce growing in the *Ledum* site than in needles of trees in the non-*Ledum* site (Table 3). Rennie (1955) found that in *Calluna*-associated soils removal of soil Ca by *Pinus sylvestris* resulted in reduced tree growth. Thus, higher foliar Ca in black spruce from the *Ledum* site may contribute to the observed growth reduction.

The *Ledum* site was different from the non-*Ledum* site in that it had higher

contents of phenolics, organic matter, acidity, moisture, and the presence of *Sphagnum*. Occurrence of *Ledum* with *Sphagnum* moss has been reported by others (Soper and Heimburger, 1985; Baldwin and Sims, 1989). High total phenolic content in organic soils amended with *Ledum* (Table 4), indicate that *Ledum* has the potential to leach water-soluble phenolics to its immediate environment. Furthermore, because of its evergreen nature, *Ledum* accumulates large amounts of organic matter, mainly leaf litter and fine roots, as does *Kalmia angustifolia* (Damman, 1971). It thus contributes substantial phenolics to the soil environment (Patrick, 1971; Rice, 1984). The high organic layer build-up results in more soil moisture content (Brady, 1990), which helps in leaching water-soluble phenolics from living *Ledum* leaves or its litter. High acidity in *Ledum* sites (Sims et al., 1989) could be due to a higher phenolic content of *Ledum* soils and the presence of *Sphagnum* moss.

Total phenolic content was higher in needles of black spruce growing in the *Ledum* site than in needles of trees in the non-*Ledum* site (Table 3). This may be due to the higher uptake of phenolics from the *Ledum* soils (Rice, 1984) or due to synthesis of more phenolics by black spruce in response to physiological stress created in the *Ledum* site (Inderjit and Dakshini, 1992; Rice, 1984; Tang et al., 1995). Phenolics have the potential to create nutrient imbalances in susceptible plants growing in the vicinity of donor plants (Einhellig, 1995; Inderjit and Dakshini, 1992; Rice, 1984). Whether the observed changes in foliar chemistry of black spruce growing in *Ledum* sites, were due to phenolics contributed by *Ledum* needs further experimentation.

The sensitivity of a plant to allelopathic compounds may be related to its age. If the susceptible plant survives until the sensitive age is crossed, it can generally overcome the adverse effects of allelopathy. In the present study, shoot height of black spruce was strongly affected in the *Ledum* site during the first seven years, but in the eighth year black spruce in the *Ledum* site showed better growth compared to the earlier years. However, even if black spruce could overcome the growth inhibition eventually, it has lost a significant part of its initial seven years of growth. This is a serious loss from the forest productivity point of view. Planting older black spruce seedlings in *Ledum* sites may minimize the adverse effects of *Ledum* sites.

In nature many factors interact both simultaneously and sequentially, and their relationships are constantly changing. There exist many collinearities such as humus increase, soil temperature decline, soil temperature increase, and phenolic content increase (Inderjit and Dakshini, 1995). The present study in no way demonstrates the allelopathic interference of *Ledum* with the growth and physiological parameters of black spruce. This study only quantifies the damage to growth and physiological variables of black spruce growing in *Ledum*-dominated sites. Preliminary data on soil chemistry indicate that allelopathy may be one of the mechanisms for these growth differences observed. The most likely

explanations for the observed growth reductions could be: (1) soil differences that could be mediated by allelopathic compounds (e.g., effects on nitrogen cycle, or phenolics could stimulate microbial activity which depletes soil nitrogen), (2) competition by *Ledum*, (3) allelopathic compounds that affect spruce-mycorrhizae symbiosis adversely, and (4) phenolics that may have direct effects on plant growth. Our future investigations will pursue the mechanism for the observed growth differences of black spruce at sites with and without *Ledum*, particularly the role of allelopathic compounds.

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ACTION OF ANTIALGAL COMPOUNDS FROM *Juncus effusus* L. ON *Selenastrum capricornutum*

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Abstract—Twenty-nine free and glucosylated dihydrophenanthrenes and tetrahydropyrenes isolated from *Juncus effusus* have been tested against the unicellular green alga *Selenastrum capricornutum*. The free compounds showed a strong inhibitory activity, while the glucosides were inactive or had a slight stimulating effect on growth. Tetrahydropyrene **33**, obtained by hydrolysis of the natural β-glucosides **29** and **30** or by hemisynthesis from **1**, is the most active compound, causing 90% inhibition of the growth at 2.5×10^{-5} M concentration. Synthetic α-glucosides **28**, **31**, and **32** showed comparable activity to those of **29** and **30**.

Key Words—*Juncus effusus*, dihydrophenanthrenes, tetrahydropyrenes, algicides, *Selenastrum capricornutum*.

INTRODUCTION

It is well known that excessive algal growth can have detrimental effects on both indigenous organisms and potential human uses of aquatic ecosystems (Lembi et al., 1988). Algae can cause poisoning of fish and livestock (Steidinger and Baden, 1984; Carmichael, 1986), as well as damage irrigation canals, drainage ditches, and other man-made structures (Hansen et al., 1984).

The need for algicides not harmful to the environment prompted us, several years ago, to screen natural substances isolated from aquatic macrophytes that

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could give useful results in the control of algal growth. Although some sterols and fatty acids toxic to algae have been found (Aliotta et al., 1990; Pinto et al., 1995), phenolic compounds have given more promising results. These chemicals are among the most frequently identified allelochemicals from higher plants and are also toxic to various microorganisms (Elliot and Cheng, 1985). Phenolic compounds active against microalgae have been isolated from aquatic and wetland plants such as *Acorus gramineus, Pistia stratiotes, Myriophyllum verticillatum,* and *Arum italicum* (Aliotta et al., 1995).

Recently, it has been shown that dihydrophenanthrenes, uncommon phenolic compounds isolated from the wetland plant, *Juncus effusus,* have a strong toxic action on microorganisms such as *Agrobacterium tumefaciens* and on small invertebrates such as *Artemia salina* (Della Greca et al., 1993). The toxicity of these aromatic compounds to algae is still unknown. In this paper we report the biological effects of a series of free and glucosylated dihydrophenanthrenes, isolated from *Juncus effusus,* on the unicellular green alga *Selenastrum capricornutum.*

METHODS AND MATERIALS

Plant Collection and Extraction. Plants of *Juncus effusus* were collected during spring and summer 1991 near Naples. A voucher specimen is on deposit at the Botanical Garden of Naples. The plants were dried at room temperature and sequentially extracted with ethyl ether (Et_2O) and methanol (MeOH). The extracts were frozen and stored at −20°C until used.

General Experimental Procedures. NMR spectra were recorded at 400 MHz for ^1H and 100 MHz for ^{13}C on a Bruker AC 400 spectrometer in $CDCl_3$ or acetone-d_6 solutions for dihydrophenanthrenes and pyrenes and in DMSO-d_6 solutions for the glycosyl derivatives. One-bond and long-range H-C COSY experiments were performed with the XHCORR microprogram using delays corresponding to $J_{C,H}$ = 160 Hz and 8 Hz, respectively. EI and FAB mass spectra were obtained with a Kratos MS 80 and a VG ZAB 2SE, respectively. IR spectra were determined in $CHCl_3$ solutions on a FT-IR Perkin-Elmer 1740 spectrometer. DCCC was run with a mixture of $CHCl_3$, MeOH, and H_2O (13:7:4 for **19, 20, 23–26,** and **29;** 26:14:5 for **27** and **30;** and 13:7:2 for **21** and **22**); the polar upper layer was used as the mobile phase. Reverse-phase HPLC was performed using LiChrosorb RP18 columns [MeOH–H_2O (9:1) for **5, 12,** and **14;** MeOH–H_2O (4:1) for **1** and **6;** MeOH–H_2O (3:1) for **2, 15,** and **18**], LiChrosorb RP8 columns [MeOH–H_2O (1:1) for **19, 20, 23–26** and **29;** MeOH–H_2O (2:3) for **21** and **22;** MeOH–H_2O (3:7) for **30**], and a LiChrosorb NH_2 column [CH_3CN–H_2O (4:1) for **27**].

Ethyl Ether Extract Fractionation. The Et_2O extract (125 g) was separated by conventional procedures into an acidic (34 g) and a neutral fraction (85 g). The neutral fraction was chromatographed on silica gel and the fractions eluted with hexane–Et_2O (9:1). Preparative TLC (hexane–benzene, 2:3) gave pure juncunol (**3**) (66 mg) (Sarkar et al., 1988). Elution with hexane–Et_2O (17:3) gave **11,** purified by preparative TLC (hexane–AcOEt, 13:7) (4 mg): IR: ν_{max} 3340 and 1603 cm^{-1}; EI-MS: *m/z* 266 $[M]^+$ (96%), 251 $[M - Me]^+$ (100), 236 $[M - 2\ Me]^+$ (52), 165 (84), 149 (39). Elution with hexane–Et_2O (4:1) gave a mixture of **7** and **8,** which were separated by preparative TLC (benzene–Et_2O, 9:1). Pure **7** had (18 mg) IR: ν_{max} 3480, 1612, and 1260 cm^{-1}; EI-MS: *m/z* 280 $[M]^+$ (76%), 265 (100), 250 (53), 237 (42). Pure **8** had (16 mg) IR: ν_{max} 3475, 1614, and 1257 cm^{-1}; EI-MS: *m/z* 280 (78%), 265 (100), 250 (44), 237 (51).

The acidic fraction was chromatographed on HCl-washed silica gel. The fractions eluted with hexane–Et_2O (17:3) were chromatographed on silica gel to give **4, 5, 12, 14,** and **16.** Compound **4** (250 mg) eluted with benzene–Et_2O (3:17) had IR: ν_{max} 3350 and 1605 cm^{-1}; EI-MS: *m/z* 266 (96%), 251 (100), 236 (45), 165 (80), 149 (50). Compound **16** eluted with benzene–Et_2O (1:19) was purified by preparative TLC (benzene–Et_2O, 17:3) (5 mg); it had IR: ν_{max} 3460, 1620, and 1140 cm^{-1}; EI-MS: *m/z* 298 (100%), 283 (80), 255 (43). The mixture of **5, 12,** and **14,** eluted with benzene–Et_2O (1:19), was resolved by preparative reverse-phase HPLC to give juncunone (**12,** 18 mg) (Miles et al., 1981), **14** (16 mg), and **5** (125 mg). Compound **14** had IR: ν_{max}: 3340, 1705, and 1620 cm^{-1}; EI-MS: *m/z* 268 (100%), 239 (51). **5** had IR: ν_{max} 3340 and 1610 cm^{-1}; EI-MS: *m/z* 266 (90%), 251 (100), 236 (40), 165 (80). The fractions eluted with hexane–Et_2O (4:1), after chromatography on silica gel (hexane–Me_2CO, 7:3) and reverse-phase HPLC, gave juncusol (**1,** 16 mg) (Shima et al., 1991) and **6** (130 mg). Compound **6** had IR: ν_{max} 3130 and 1605 cm^{-1}; EI-MS: *m/z* 266 (85%), 251 (100), 236 (70). The fractions eluted with benzene–Et_2O (7:3) were chromatographed on silica gel. Benzene–Et_2O (19:1) gave effusol (**2,** 64 mg) (Shima et al., 1991), purified by reverse-phase HPLC, and **13** (13 mg), purified by preparative TLC [hexane–acetone (Me_2CO), 7:3]. Compound **13** had IR: ν_{max} 3350, 1705, 1620, and 1603 cm^{-1}; EI-MS: *m/z* 282 (70%), 267 (100), 253 (45). Benzene–Et_2O (4:1) gave a mixture of **18** and **15** and a mixture of **9** and **10.** The first mixture was resolved by reverse-phase HPLC to give micrandol B (**18,** 7 mg) (De Alvarenga and Gottlieb, 1974) and **15** (40 mg). Compound **15** had IR: ν_{max} 3340 and 1605 cm^{-1}; EI-MS: *m/z* 284 (100%), 266 (68). The latter mixture was separated by preparative TLC (benzene–Et_2O–AcOH, 60:31:1) to give **9** (8 mg): IR: ν_{max} 3520, 1690, and 1610 cm^{-1}; EI-MS: *m/z* 280 (100%), 273 (65), 235 (55) and **10** (10 mg): IR: ν_{max} 3500, 1685, and 1620 cm^{-1}; EI-MS: *m/z* 280 (100%), 273 (72), 235 (41). The

fractions eluted with hexane–Et_2O (3:2) gave, after preparative TLC (benzene–Et_2O, 1:3), **17** (22 mg): IR: ν_{max} 3350 and 1610 cm^{-1}; EI-MS: *m/z* 284 (100%), 266 (75).

Methanol Extract Fractionation. The MeOH extract (350 g), after removal of the solvent, was partitioned between ethyl acetate and water. The organic layer was chromatographed on silica gel and the fractions eluted with $CHCl_3$–MeOH (9:1) were chromatographed on Sephadex LH-20 (MeOH–H_2O, 1:1). DCCC and HPLC of fractions 85–116 gave pure **19** (6 mg): FAB-MS: *m/z* 428; **20** (12 mg): FAB-MS: *m/z* 428; and **29** (15 mg): FAB-MS: *m/z* 428. DCCC chromatography and HPLC chromatography of fractions 130–143 gave pure **23** (10 mg): FAB-MS: *m/z* 446; **24** (8 mg): FAB-MS: *m/z* 432; **25** (11 mg): FAB-MS: *m/z* 432; and **26** (6 mg): FAB-MS: *m/z* 432. The aqueous layer was chromatographed on Amberlite X-AD2 and the MeOH fraction was chromatographed on Sephadex LH-20 (MeOH–H_2O, 1:3). Subsequent DCCC and HPLC processes gave pure **21** (11 mg): FAB-MS: *m/z* 590; **22** (14 mg): FAB-MS: *m/z* 590; **27** (11 mg): FAB-MS: *m/z* 608; and **30** (8 mg): FAB-MS: *m/z* 590.

Synthesis of Compounds 28, 31, 32, and 33. Pure 2,7-dihydroxy-1,6-dimethyl-5-vinyl-9,10-dihydrophenanthrene (**1**, 400 mg) was dissolved in EtOH (10 ml) and 2 N NaOH (20 ml) and Me_2SO_4 (4 ml) were added. After 18 hr with magnetic stirring the reaction mixture was extracted with ethyl ether and the crude methoxy derivative of **1** (**1m**) was purified by preparative TLC (hexane–benzene, 1:1). A solution of the methoxy derivative **1m** (240 mg) in benzene (200 ml) was irradiated with a 450-W Hanovia lamp equipped with a Pyrex filter sleeve for 1.5 hr under N_2. After removal of the solvent, the reaction mixture was chromatographed by preparative TLC (hexane–benzene, 1:1) to give pure 2,7-dimethoxy-1,6-dimethyl-9,10,12,13-tetrahydropyrene (**33m**) (200 mg): ^1H NMR: δ ($CDCl_3$) 6.65 (s, H-3 and H-8), 2.86 (s, H-9, H-10, H-12 and H-13), 2.22 (s, H-11 and H-14), 3.88 (s, OMe); ^{13}C NMR: δ 121.5 (C-1 and C-6), 156.2 (C-2 and C-7), 107.8 (C-3 and C-8), 132.6 (C-4 and C-8a), 134.8 (C-5 and C-1a), 28.9 (C-9 and C-13), 25.1 (C-10 and C-12), 11.2 (C-11 and C-14), 124.1 (C-4a and C-5a), 55.7 (OMe).

A solution of BBr_3 (1 M) in CH_2Cl_2 (300 μl) was added to a solution of pure **33m** (180 mg) in dry CH_2Cl_2 (9 ml) at −70°C under N_2. After 4 hr at 0°C with magnetic stirring, H_2O (6 drops) and sat'd. aq. $NaHCO_3$ (6 ml) were added and the mixture was extracted with EtOAc to give crude 2,7-dihydroxy-1,6-dimethyl-9,10,12,13-tetrahydropyrene **33** (170 mg), which was purified by preparative TLC (benzene–Et_2O, 7:3), and had ^1H NMR: δ 6.53 (s, H-3 and H-8), 2.64 (s, H-9, H-10, H-12, and H-13), 2.07 (s, H-11 and H-14); ^{13}C NMR: δ 118.7 (C-1 and C-6), 153.3 (C-2 and C-7), 111.7 (C-3 and C-8), 131.7 (C-4 and C-8a), 134.0 (C-5 and C-1a), 28.1 (C-9 and C-13), 24.6 (C-10 and C-12), 11.2 (C-11 and C-14), 122.6 (C-4a and C-5a).

Tetrahydropyrene (**33,** 135 mg, 0.5 mmol) in dry CH_3CN (10 ml) was treated with $Hg(CN)_2$ (1.5 mmol) and acetobromo-α-D-glucose (1 mmol). After 24 hr at room temperature the mixture was diluted with $CHCl_3$ and filtered. The filtrate was chromatographed on silica gel to give crude peracetates of **28** (hexane–EtOAc, 7:3, 31 mg) and **29** (hexane–EtOAc, 3:2, 65 mg) and a mixture of peracetates of **30, 31,** and **32** (hexane–EtOAc, 1:1, 60 mg). The peracetates of **28** and **29** were heated 30 min with refluxing ethanolic KOH (5%) and the glucosides **28** and **29** were extracted with *n*-BuOH and purified by HPLC. Compound **28** (22 mg) had ^{1}H NMR: δ (CD_3OD) 6.95 (s, H-3), 6.51 (s, H-8), 5.48 (d, J = 2.8 Hz, G-1), 2.75 (s, H-9, H-10, H-12, H-13), 2.25 (s, H-11), 2.14 (s, H-14).

The mixture of peracetates was resolved by preparative TLC (benzene–Et_2O, 3:2), and the pure compounds were hydrolyzed with ethanolic KOH (5%) to give **30** (8 mg), **31** (13 mg), and **32** (9 mg). Compound **31** had ^{1}H NMR: δ (CD_3OD) 6.92 (s, H-3), 6.97 (s, H-8), 5.50 (d, J = 2.8 Hz, G-1β), 4.88 (overlapped G-1α), 2.78 (s, H-9, H-10, H-12, H-13), 2.24 (s, H-11), 2.26 (s, H-14). Compound **32** had ^{1}H NMR: δ (CD_3OD) 6.97 (s, H-3 and H-8), 5.51 (d, J = 2.8 Hz, G-1), 2.78 (s, H-9, H-10, H-12, H-13), 2.26 (s, H-11 and H-14).

Bioassay. The strain UTEX 1648 *Selenastrum capricornutum* was maintained on Bold basal medium (BBM) (Nichols, 1973) solidified with agar 1.5% in continuous light at 23°C. Fresh axenic cultures for the experiments were grown in 100-ml cylinders on the same culture medium. For the growth tests the phenanthrenes **1–18** and **33** were dissolved in acetone, compounds **21, 22, 27,** and **30–32** in water, and the remaining compounds in DMSO. Each solution (20 μl) was added to the test tubes containing 6 ml of inoculated medium, to give final concentrations of 10^{-4}, 7.5×10^{-5}, 5×10^{-5}, 2.5×10^{-5}, and 10^{-5} M. Blanks containing only acetone, DMSO, or water in BBM, respectively, were also tested. The test tubes were incubated at 23°C on a shaking apparatus previously described (Aliotta et al., 1991). The total irradiation of 150 μE/sec m^2 was provided by daylight fluorescent lamps (Philips TLD 30 w/55) from below the apparatus. The photoperiod was 16 hr light–8 hr dark. Growth of cultures was followed daily either by measuring the absorbance increase at 550 nm with a Bausch & Lomb Spectronic 20 colorimeter or by counting the cell numbers with a Thoma blood-counting chamber. The cell numbers of the initial inocula ranged from 10^6 to 1.5×10^6/ml, corresponding to 0.05–0.06 units of absorbance. Growth experiments were carried out in triplicate. According to the two types of growth inhibition observed (reduced growth rate for compounds **1, 2,** and **4–32** and increased lag phase for **3** and **33**), the growth rates or the lag phases were calculated. To test statistical significance of results, one-way ANOVA was performed at $\alpha = 0.05$. For each

compound, a comparison among means was performed using Student-Newman-Keuls test (SNK), at $\alpha = 0.05$. The SPSS statistical package was used.

The index of inhibition for compounds **1, 2,** and **4–32** was calculated as: $(1 - X_a/Y_a) \times 100$ (Blankley, 1973), where X_a is the growth rate of the alga in the presence of the compound tested, and Y_a is the growth rate of the control. The inhibition for compounds **3** and **33** was calculated as: $(1 - X_b/Y_b) \times 100$ (Blankley, 1973), where X_b is the lag phase of the alga in the presence of the compounds tested, and Y_b is the lag phase of the control.

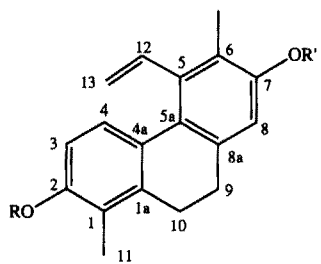
RESULTS AND DISCUSSION

The structures of the compounds tested in this study are shown in Figure 1. Plants of *J. effusus* were air-dried and extracted with ethyl ether and methanol sequentially. The crude extracts were assayed for their biological effects on *S. capricornutum,* one of the organisms recommended for the standard algal assay procedure developed for aquatic systems (Pipe and Shubert, 1984). Both extracts were effective in inhibiting algal growth. The ethyl ether extract was separated into an acidic and a neutral fraction and sequential bioassay-oriented chromatographic processes (CC, TLC, and HPLC) of these fractions gave dihydrophenanthrenes **1–18,** which were mainly identified on the basis of their ^{13}C NMR (Tables 1 and 2) and ^{1}H NMR data (Tables 3 and 4) (Della Greca et al., 1992, 1993). The bioassay-oriented chromatographic processes (CC, DCCC, and HPLC) of the methanolic extract gave 11 glycosides, which were identified as dihydrophenanthrene glucosides **19–27** and tetrahydropyrene glucosides **29** and **30** on the basis of their spectroscopic features (Tables 2, 4, and 5) (Della Greca et al., 1995a–c).

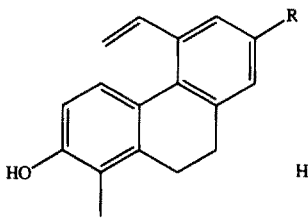
The natural compounds **1-27, 29,** and **30** and the correlated hemisynthetic tetrahydropyrenes **28, 31–33** were assayed in broth against *S. capricornutum.* Two types of inhibition were observed. Compounds **1, 2,** and **4–32** caused a decrease of the growth rate during the exponential phase of growth, while in the experiments with **3** and **33** an increase of the lag phase, with lengthening dependent on the initial concentration of the compounds, was observed. The activity was calculated as percentage of inhibition or stimulation caused by compounds at five different concentrations (Tables 6 and 7).

All the free dihydrophenanthrenes inhibited algal growth. Compounds **1, 4, 6, 12, 15,** and **16** were lethal to the alga at 10^{-4} M, the highest concentration used, and 13 compounds (**1, 4, 7–13** and **15–18**) caused 15–30% inhibition at 10^{-5} M, the lowest concentration tested.

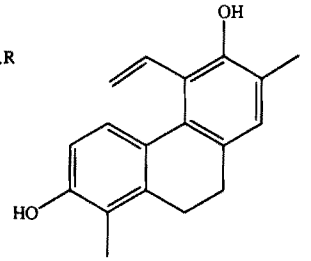
A comparison among compounds **1, 2,** and **5,** which had identical patterns of substituents at C-1, C-2, C-5, and C-7, showed that the presence of the



1 R = R' = H
1m R = R' = Me
19 R = β-D-glc R' = H
20 R = H R' = β-D-glc
21 R = R' = β-D-glc



2 R = OH
3 R = Me



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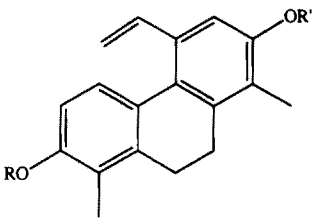
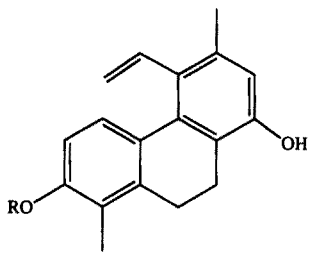
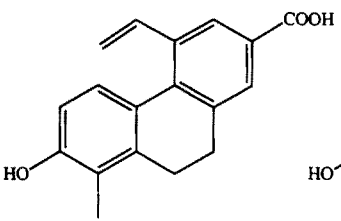


5 R = R' = H
8 R = H R' = Me
22 R = R' = β-D-glc



6 R = H
7 R = Me



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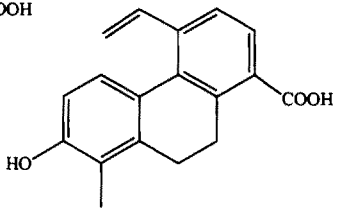

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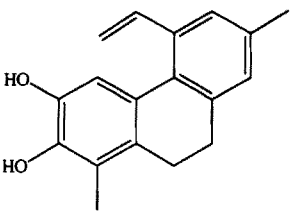

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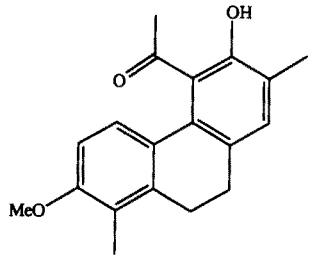
FIG. 1. Structures of compounds reported in this research.



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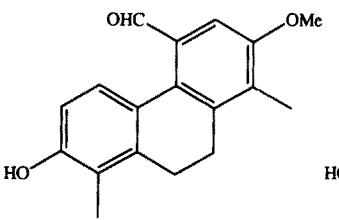

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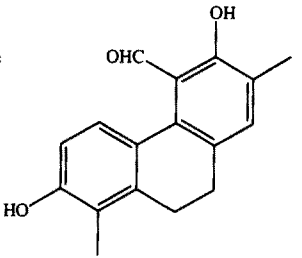

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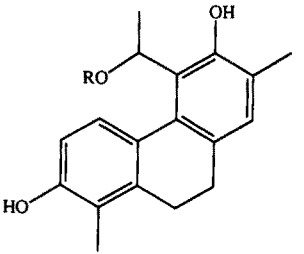
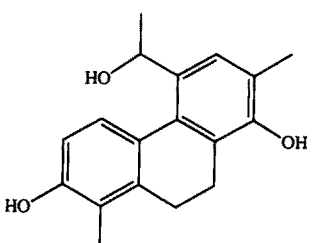


15 R = H
16 R = Me



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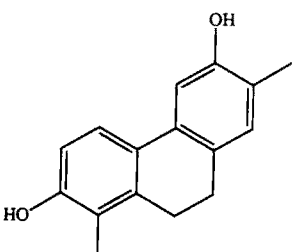

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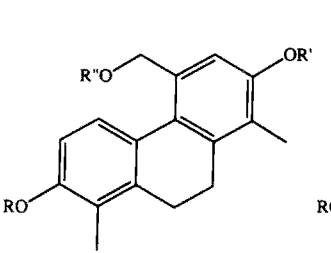
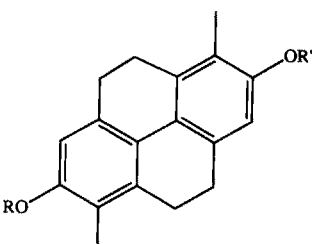


23 R = H R' = Me R" = β-D-glc
24 R = R' = H R" = β-D-glc
25 R = R" = H R' = β-D-glc
26 R = β-D-glc R' = R" = H
27 R = R" = β-D-glc R' = Me



28 R = α-D-glc R' = H
29 R = β-D-glc R' = H
30 R = R' = β-D-glc
31 R = β-D-glc R' = α-D-glc
32 R = R' = α-D-glc
33 R = R' = H
33m R = R' = Me

FIG. 1. Continued.

TABLE 1. ^{13}C NMR OF DIHYDROPHENANTHRENES **1–15**

C	1[a]	2[b]	3[b]	4[b]	5[b]	6[a]	7[b]	8[b]	9[a]	10[a]	11[b]	12[b]	13[b]	14[a]	15[a]
1	121.0	120.5	120.6	120.7	120.5	121.0	120.4	120.7	122.1	121.9	121.9	121.3	121.3	122.7	122.1
2	154.2	153.8	152.6	152.7	152.1	154.2	155.9	153.1	156.5	156.6	150.5	155.9	156.4	156.9	155.3
3	112.0	111.8	111.8	111.6	111.8	111.9	106.7	111.7	112.7	114.0	140.4	112.6	112.4	113.1	112.7
4	128.8	127.6	127.6	127.6	127.9	128.7	128.1	128.1	128.6	124.0	118.9	128.5	129.4	130.3	126.3
5	137.8	136.8	135.0	120.0	133.6	127.5	127.5	133.6	135.5	138.8	135.2	120.2	133.4	116.9	125.2
6	121.6	112.9	127.3	149.6	112.0	137.2	137.5	107.5	125.5	122.7	127.7	154.2	106.2	160.5	148.9
7	154.5	154.4	135.8	122.4	152.3	113.7	113.0	156.1	128.6	128.9	136.0	124.4	154.1	123.9	126.1
8	113.7	114.4	128.0	129.1	120.3	154.4	152.3	117.9	128.7	129.4	127.5	133.9	129.9	137.4	129.3
9	31.1	30.3	30.0	29.2	26.3	31.0	30.3	26.3	29.2	26.9	29.9	31.8	25.5	28.7	30.6
10	26.4	25.5	25.6	26.0	25.3	26.3	25.7	25.5	25.6	25.7	25.6	28.2	25.2	26.2	26.8
11	11.9	11.7	11.7	11.7	11.6	11.8	11.7	11.6	11.9	11.5	12.2	11.6	11.5	11.8	11.9
12	139.1	138.5	139.0	136.9	138.6	139.0	137.6	139.3	139.4	136.6	138.7	207.9	193.2	197.7	69.1
13	119.1	113.9	113.8	118.5	113.0	119.1	119.5	112.9	114.9	119.1	114.5	25.5			25.0
1a	140.0	139.4	139.9	140.2	139.2	140.0	139.3	139.4	139.8	140.0	129.1	139.0	139.4	140.9	141.2
4a	127.4	127.1	127.0	127.4	127.4	127.3	127.3	127.4	127.6	126.7	127.2	127.8	124.7	124.0	124.9
5a	127.5	127.6	131.9	131.9	127.6	137.7	136.9	127.5	141.4	135.2	130.7	135.0	131.3	139.0	133.6
8a	137.2	139.4	138.7	129.6	139.2	121.5	122.5	138.9	139.4	139.2	138.5	129.5	139.3	130.1	131.4
Me	13.7		21.1	16.3	11.5	13.6	13.2	11.5			21.1	15.8	12.3	15.1	15.6
OMe							55.5	55.6				55.7			56.7
COOH									167.5	169.3					

[a]Recorded in acetone-d_6.
[b]Recorded in $CDCl_3$.

TABLE 2. ^{13}C NMR OF DIHYDROPHENANTHRENES **16–27** AND TETRAHYDROPYRENES **29** AND **30**

C	16[b]	17[a]	18[a]	19[c]	20[c]	21[c]	22[c]	23[c]	24[c]	25[c]	26[c]	27[c]	29[c]	30[c]
1	120.9	122.1	122.1	120.6	120.1	123.3	123.5	121.4	120.9	122.5	123.0	123.1	122.1	122.2
2	153.6	155.4	155.2	153.8	153.8	154.1	154.6	154.0	153.7	153.9	154.0	154.3	153.8	154.5
3	111.6	112.3	113.6	111.3	111.1	111.4	111.9	111.9	111.9	111.7	111.9	112.0	112.8	112.8
4	128.8	127.2	130.6	127.4	127.6	127.8	127.3	126.4	126.2	126.3	125.9	126.5	132.2	132.6
5	122.1	130.6	110.0	136.8	135.6	137.0	132.7	130.9	131.1	135.7	135.8	131.6	133.6	134.5
6	152.8	128.9	155.1	123.1	123.5	123.8	112.3	111.0	115.5	115.1	114.3	111.0	118.8	122.2
7	126.9	127.0	122.4	153.7	153.8	154.2	154.6	155.0	156.2	153.6	153.5	155.4	153.7	154.5
8	126.4	155.6	122.3	112.8	113.4	113.4	123.9	119.8	120.2	120.0	120.8	121.5	111.7	112.8
9	29.9	30.7	29.9	29.9	30.1	30.1	25.9	25.0	24.9	25.0	25.1	25.0	27.9	28.3
10	26.4	27.0	26.2	25.3	25.2	25.2	25.0	25.9	26.0	26.0	25.9	25.9	24.3	24.6
11	11.8	11.9	11.6	11.9	11.6	12.0	11.9	11.5	11.6	11.6	11.5	11.8	11.3	11.5
12	58.9	68.6		137.8	137.6	137.6	138.6	68.9	69.4	62.2	62.0	68.7	24.3	24.6
13	20.9	22.9		119.0	118.9	119.4	113.9	138.3	138.2	138.5	138.0	138.3	28.3	28.3
1a	141.1	141.8	137.9	138.6	139.0	139.0	138.8	137.9	138.2	138.5	138.0	138.3	134.5	134.5
4a	124.5	124.3	127.3	125.0	125.2	127.7	127.3	128.5	128.0	129.1	128.5	127.8	125.1	124.8
5a	133.3	132.8	134.5	128.4	128.1	128.0	128.0	125.0	127.1	125.4	126.9	127.9	122.0	124.8
8a	130.3	126.2	127.4	136.3	136.4	136.2	138.0	138.3	138.2	138.5	138.0	138.3	131.7	132.6
Me	15.7	15.8	15.8	13.4	13.6	13.6	11.9	11.5	11.6	11.8	11.8	11.5	11.0	11.5
OMe								55.4				55.5		
glc1				101.3		101.3	101.6	101.6	101.1	101.6	101.3	101.6	101.5	101.7
2				73.4		73.4	73.4	73.5	73.5	73.4	73.4	73.5	73.2	73.6
3				77.0		77.0	77.2	76.8	76.8	77.0	77.0	76.9	76.8	77.2
4				69.7		69.8	69.9	70.1	70.1	69.7	69.8	69.7	69.5	69.9
5				76.5		76.6	76.7	76.6	76.8	76.7	76.6	76.6	76.3	76.8
6				60.8		60.8	60.9	61.1	61.1	60.8	60.8	60.7	60.5	60.9
glc1′					101.4	101.4	101.3					101.2		
2′					73.4	73.4	73.4					73.4		
3′					77.0	77.0	77.2					76.9		
4′					69.8	69.8	69.9					70.0		
5′					76.7	76.6	76.7					76.6		
6′					60.8	60.8	60.9					61.0		

TABLE 3. ^{1}H NMR OF DIHYDROPHENANTHRENES **1–10**

H	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[b]	**6**[a]	**7**[b]	**8**[b]	**9**[a]	**10**[a]
3	6.62d	6.68d	6.70d	6.67d	6.66d	6.67d	6.72d	6.69d	6.82d	6.85d
	(8.5)[c]	(8.4)	(8.3)	(8.7)	(8.5)	(8.5)	(8.8)	(8.6)	(8.4)	(8.4)
4	7.54d	7.32d	7.39d	7.54d	7.25d	7.43d	7.64d	7.29d	7.38d	7.55d
	(8.5)	(8.4)	(8.3)	(8.7)	(8.5)	(8.5)	(8.8)	(8.6)	(8.4)	(8.4)
6		6.90d	7.25s		6.87s			6.95s	8.07d	7.78d
		(2.7)							(1.4)	(8.2)
7						6.72s				7.67d
										(8.2)
8	6.68s	6.71d	7.03s	6.99s			6.68s		7.83d	
		(2.7)							(1.4)	
9	2.68m	2.72m	2.74m	2.68m	2.69brs	2.58m	2.70m	2.72brs	2.82m	2.83m
10	2.68m	2.72m	2.74m	2.68m	2.69brs	2.58m	2.70m	2.72brs	2.82m	2.83m
11	2.29s	2.27s	2.28s	2.26s	2.26s	2.20s	2.30s	2.28s	2.25s	2.20s
12	6.78dd	6.92dd	6.98dd	6.73dd	6.90dd	6.74dd	6.81dd	6.99dd	6.98dd	7.19dd
	(11.3 17.9)	(10.9 17.5)	(10.8 17.4)	(11.6 18.5)	(10.8 17.4)	(11.3 18.0)	(11.4 18.0)	(10.8 17.4)	(11.0 17.6)	(11.6 17.8)
13	5.51dd	5.27dd	5.27dd	5.69dd	5.16dd	5.44dd	5.54dd	5.24dd	5.22dd	5.48dd
	(1.9 11.3)	(1.5 10.9)	(1.5 10.8)	(1.7 11.6)	(1.2 10.8)	(2.1 11.3)	(2.0 11.4)	(1.4 10.8)	(1.4 10.8)	(2.0 11.6)
	5.23dd	5.68dd	5.71dd	5.76dd	5.58dd	5.16dd	5.27dd	5.71dd	5.65dd	5.13dd
	(1.9 17.9)	(1.5 17.5)	(1.5 17.4)	(1.7 18.5)	(1.2 17.4)	(2.1 18.0)	(2.0 18.0)	(1.4 17.4)	(1.4 17.6)	(2.0 17.8)
Me	2.25s		2.38s	2.30s	2.30s	2.16s	2.24s	2.23s		
OMe						3.86s	3.91s			

[a]Recorded in acetone-d_6.
[b]Recorded in $CDCl_3$.
[c]The couplings (Hz) are reported in parantheses.

TABLE 4. ^{1}H NMR OF DIHYDROPHENANTHRENES **11–20**

H	**11**[b]	**12**[b]	**13**[b]	**14**[a]	**15**[b]	**16**[b]	**17**[a]	**18**[a]	**19**[c]	**20**[c]
3		6.68d	6.77d	6.76d	6.81d	6.71d	6.77d	6.75d	6.90d	6.63d
		(8.2)[d]	(8.4)	(8.3)	(8.4)	(8.4)	(8.6)	(9.0)	(8.7)	(8.5)
4	7.46s	6.84d	6.86d	6.94d	7.21d	7.01d	7.07d	7.60d	7.42d	7.34d
		(8.2)	(8.4)	(8.3)	(8.4)	(8.4)	(8.6)	(9.0)	(8.7)	(8.5)
5								7.10s		
6	7.23s		7.36s				6.90s			
7										
8	7.05s	7.17s		7.30s	7.00s	7.00s		6.88s	6.69s	6.98s
9	2.80m	2.75m	2.80m	2.73m	2.84s	2.72s	2.83s	2.68s	2.60m	2.61m
10	2.80m	2.75m	2.80m	2.73m	2.84s	2.72s	2.83s	2.68s	2.60m	2.61m
11	2.35s	2.28s	2.30s	2.29s	2.21s	2.27s	2.21s	2.16s	2.19s	2.20s
12	6.99dd		9.70s	9.95s	5.73q	5.14d	5.63q		6.77dd	6.77dd
	(10.8 17.3)				(6.3)	(6.2)	(6.3)		(11.5 18.0)	(11.4 18.4)
13	5.22dd	2.08s			1.43d	1.76d	1.65d		5.11dd	5.10dd
	(1.4 10.8)				(6.3)	(6.2)	(6.3)		(2.1 18.0)	(1.5 18.4)
	5.65dd								5.46dd	5.43dd
	(1.4 17.3)								(2.1 11.5)	(1.5 11.4)
Me	2.20s	2.38s	2.32s	2.30s	2.21s	2.27s	2.16s	2.16s	2.12s	2.09s
OMe			3.93s			3.21s				
glc1									4.78d	4.80d
									(7.6)	(7.5)

[a]Recorded in acetone-d_6.
[b]Recorded in $CDCl_3$.
[c]Recorded in CD_3OD.
[d]The couplings (Hz) are reported in parentheses.

methyl group in the C ring enhanced the activity. Compound **1** with the methyl at C-6 was more active than compound **5** with the same substituent at C-8.

Less important is the presence of the polar hydroxyl group in the C ring. Compound **3,** which lacks this group, and compound **8,** which has a methoxyl group, exhibited high activity. The isomeric compounds **1, 4, 5, 6,** and **11** as well as the isomeric carboxylic acids **9** and **10** and the isomeric carbinols **15** and **17** had rather similar activity showing that the relative positions of substituents is unimportant.

The presence of a substituent at C-5 seems to be important for biological activity. Compound **18,** which has a hydrogen at this position, is less active than compounds **4, 12, 15,** and **16.** The activity of aldehyde **14** is not comparable because of its instability in the bioassay conditions.

Glucosylated dihydrophenanthrenes **19–27** did not inhibit the algal growth and compounds **26** and **27** had a slight stimulating effect. These results are in

TABLE 5. ^{1}H NMR OF DIHYDROPHENANTHRENES **21–27** AND TETRAHYDROPYRENES **29** AND **30**[a]

H	21	22	23	24	25	26	27	29	30
3	6.90d	7.01d	6.76d	6.68d	6.71d	6.97d	7.00d	6.85s	6.84s
	(8.6)[b]	(8.6)	(8.4)	(8.3)	(8.3)	(8.8)	(8.7)		
4	7.45d	7.18d	7.38d	7.31d	7.33d	7.37d	7.43d		
	(8.6)	(8.6)	(8.4)	(8.3)	(8.3)	(8.8)	(8.7)		
6		7.23s	7.14s	6.88s	7.18s	6.94s	7.16s		
8	7.00s							6.54s	6.84s
9	2.60m	2.69m	2.62m	2.62m	2.62m	2.62m	2.54m	2.66s	2.70s
							2.63m		
10	2.60m	2.69m	2.62m	2.62m	2.62m	2.62m	2.54m	2.68s	2.70s
							2.63m		
11	2.20s	2.20s	2.18s	2.14s	2.19s	2.21s	2.23s	2.15s	2.13s
12	6.79dd	6.83dd	4.68d	4.45d	4.48s	4.47s	4.70d	2.66s	2.70s
	(11.4 18.1)	(10.8 17.3)	(11.0)	(10.7)			(11.0)		
			4.91d	4.80d			4.88d	2.68s	2.70s
			(11.0)	(10.7)			(11.0)		
13	5.09dd	5.79dd							
	(1.7 18.1)	(1.2 17.3)							
	5.45dd	5.22dd							
	(1.7 11.4)	(1.2 10.8)							
Me	2.20s	2.23s	2.17s	2.12s	2.13s	2.11s	2.15s	2.07s	2.13s
OMe			3.85s				3.82s		
glc1	4.79d	4.80d	4.32d	4.30d	4.82d	4.79d	4.29d	4.78d	4.78d
	(7.4)	(7.3)	(7.7)	(7.8)	(7.3)	(7.3)	(7.6)	(7.6)	(7.3)
glc1′	4.81d	4.81d					4.82d		4.78d
	(7.5)	(7.4)					(7.1)		(7.3)

[a]Recorded in CD_3OD. [b]The couplings (Hz) are reported in parentheses.

agreement with the well-known detoxification process of phenolic compounds in plants occurring by glucosylation (Harborne, 1977).

Among tetrahydropyrenes, a similar pattern of inhibition was observed. Unnatural tetrahydropyrene **33,** obtained by hemisynthesis from **1,** was the most active compound tested, causing 90% inhibition of growth at 2.5×10^{-5} M. Glucosylation of the hydroxyl groups as in **28–32** drastically reduced the biological activity, and the activities of the natural β-glucosyl derivatives **29** and **30** were comparable to those of the synthetic α-derivatives **28, 31,** and **32.**

Although the reduction of nutrient efflux in the environment remains the most effective method for the control of the algal growth, mainly in the case of filamentous algae (Canale and Auer, 1982; Lembi et al., 1988), the use of algicides could be important in those sites where nutrient reduction is not readily

TABLE 6. ACTIVITY OF DIHYDROPHENANTHRENES **1–27** ON *Selenastrum capricornutum*[a]

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
10^{-4} M	100e	15d	90e	100f	64e	100e	94f	94f	90f	92f	90f	100f	98f	55e
7.5×10^{-5} M	70d	12c	72d	94e	50d	48d	80e	80e	78e	74e	82e	85e	82e	48d
5.0×10^{-5} M	65d	8b	46c	60d	34c	25c	65d	64d	60d	65d	64d	75d	72d	25c
2.5×10^{-5} M	50c	0a	15b	50c	28b	20b	50c	50c	53c	55c	50c	60c	59c	10b
10^{-5} M	15b	0a	0a	30b	0a	0a	20b	15b	22b	20b	15b	30b	28b	0a
Control	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a

	15	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
10^{-4} M	100f	100f	93e	75f	0a	0a	0a	0a	15b	0a	0a	+15b	+25b
7.5×10^{-5} M	89e	90e	71e	68e	0a	0a	0a	0a	8b	0a	0a	+12b	+25b
5.0×10^{-5} M	80d	80d	60d	50d	0a	0a	0a	0a	0a	0a	0a	0a	+20b
2.5×10^{-5} M	70c	70c	50c	35c	0a	0a	0a	0a	0a	0a	0a	0a	+20b
10^{-5} M	30b	28b	22b	20b	0a	0a	0a	0a	0a	0a	0a	0a	+20b
Control	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a

[a]Percent of inhibition or stimulation (+) with respect to the blank. In each column values followed by different letters are statistically significant; Student-Newman-Keuls test; $P = 0.05$.

applicable, such as irrigation and drainage channels that collect water from crops.

On the other hand, the search for new compounds toxic to algae has not been very successful. According to Milne and Callow (1985), there is a conflict between the need for a powerful, selective algicide and the requirement of using a safe and harmless compound. At the present time only a few algicides are effective in the control of algal growth in fresh water. Anderson and Dechoretz

TABLE 7. ACTIVITY OF TETRAHYDROPYRENES ON *Selenastrum capricornutum*

	28	**29**	**30**	**31**	**32**	**33**
10^{-4} M	40d	48c	40d	15c	0a	100d
7.5×10^{-5} M	15c	25b	30c	10b	0a	100d
5.0×10^{-5} M	10b	25b	18b	0a	0a	95c
2.5×10^{-5} M	0a	0a	0a	0a	0a	90b
10^{-5} M	0a	0a	0a	0a	0a	0a
Control	0a	0a	0a	0a	0a	0a

[a]Index of inhibition (%) with respect to the blank. In each column values followed by different letters are statistically significant; Student-Newman-Keuls test; $P = 0.05$.

(1984) indicated six active ingredients currently used in the United States for algal control: copper sulfate, simazine, endothall, acrolein, diquat, and chlorine. Copper sulfate is less toxic and a more commonly utilized algicide; it can be used in water for fishing, aquaculture, irrigation, and swimming, although its harmful effects on aquatic fauna preclude its use in large-scale treatments in natural environments (Andrews, 1986). On the other hand, this salt can have little effect on the algal population to be controlled when a dense growth occurs, especially if organic chelators are present in water (McKnight, 1981). Moreover, Elder and Horne (1978) pointed out that alkaline pH and high organic carbon levels in the water may detoxify the copper ions, so that it may actually stimulate growth.

There is evidence that certain phenolic compounds are selectively toxic to algae. Recently, a study carried out on 22 compounds that mimic natural phenylpropanoids revealed that some of these substances have a selective antialgal activity (Della Greca et al., 1995d). The differential resistance to phenylpropanoid seems to reflect ultrastructural and physiological differences within algae. A previous study provides evidence that the ultrastructure of the cell wall in green algae influences the toxic action of α-asarone. In fact, when a trilaminar cell wall with a sporopollenin layer was present, as in strain C202.7a *Ankistrodesmus braunii*, the toxic effect of α-asarone was very reduced (Pollio et al., 1993).

Previous results have indicated that the action of phenanthrenes also can be selective. Dihydrophenanthrenes **1–18** have been tested for their cytotoxic activity against brine shrimp, *Artemia salina* (Della Greca et al., 1993). It is noteworthy that some of these compounds, such as **12** and **13**, were strongly active against *S. capricornutum* and ineffective against *A. salina* at the same concentrations. Further investigations are in progress to ascertain whether dihydrophenanthrenes and tetrahydropyrenes are able to selectively control the growth of different algal strains.

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MANDIBULAR GLAND COMPONENTS OF EUROPEAN AND AFRICANIZED HONEY BEE QUEENS (*Apis mellifera* L.).

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Abstract—The composition of the five-component honey bee queen mandibular gland pheromone (QMP) of mated European honey bee queens was compared to those of virgin and drone-laying (i.e., laying only haploid unfertilized eggs that develop into males), European queens and Africanized mated queens. QMP of mated European queens showed significantly greater quantities of individual components than all queen types compared, except for a significantly greater quantity of 9-hydroxy-(*E*)-2-decenoic acid (9-HDA) found in Africanized queens. Glands of European drone-laying queens contained quantities intermediate between virgin and mated queens, reflecting their intermediate reproductive state and age. QMP ontogeny shifts from a high proportion of 9-keto-(*E*)-2-decenoic acid (ODA) in young unmated queens to roughly equal proportions of ODA and 9-HDA in mated queens. A biosynthetic shift occurs after mating that results in a greater proportion of 9-HDA, methyl *p*-hydroxybenzoate (HOB), and 4-hydroxy-3-methoxyphenylethanol (HVA) production, accompanied by a decreased proportion of ODA. Africanized QMP proportions of ODA and 9-HDA were significantly different from European queens. A quantitative definition of a "queen equivalent" of QMP is proposed for the various queen types, and a standard queen equivalent for mated European honeybee queen mandibular gland pheromone is adopted as 200 μg ODA, 80 μg 9-HDA, 20 μg HOB, and 2 μg HVA.

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Key Words—*Apis mellifera*, honey bee, queen mandibular gland pheromone, Africanized honey bee.

INTRODUCTION

One of the most interesting aspects of honey bee biology is the variability found within and between races of *Apis mellifera*. Bees vary in behavioral, morphological, and physiological characteristics. Much of the worldwide success of this species is attributed to the variation among colony members providing adaptive advantages in different habitats (Seeley, 1985; Winston, 1987, 1992). A great deal is known about the phenotypic and genotypic variability among worker bees within colonies, but the variability among queen honey bees is not as widely studied. Here we present a detailed characterization and comparison of inter- and intraracial variation of queen mandibular gland pheromone.

Queen mandibular gland pheromone (QMP) is a blend of the three abundant aliphatic acids, 9-keto-(*E*)-2-decenoic acid (ODA), and 9-hydroxy-(*E*)-2-decenoic acid (9-HDA), present as two optical isomers [*R*-(−) and *S*-(+)], and two aromatic compounds; methyl *p*-hydroxybenzoate (HOB), and 4-hydroxy-3-methoxyphenylethanol (HVA) (Slessor et al., 1988). As a primer pheromone, QMP has been shown to delay swarming (Winston et al., 1991), suppress queen rearing (Pettis et al., 1995; Winston et al., 1989, 1990), inhibit worker juvenile hormone (JH) biosynthesis in the laboratory (Gast, 1967; Hildebrandt and Kaatz, 1990; Kaatz et al., 1992), and regulate foraging age of adult worker bees and suppress JH titers in colonies (Pankiw et al., unpubl. data). QMP also has some releaser pheromone functions, including retinue formation around the queen (Slessor et al., 1988; Kaminski et al., 1990), attraction of workers to swarms (Winston et al., 1989), stimulation of pollen foraging (Higo et al., 1992), calming of queenless workers (Naumann et al., 1990), and attraction of foragers to crops (Currie et al., 1992a,b).

In this study we extracted QMP from the glands of various European and Africanized queen types and compared the quantities and ratios of individual components. The ontogenetic development of the QMP signal also is presented, and a definition of one queen equivalent is proposed that reflects racial and reproductive differences, while allowing for the natural variation found among individuals.

The only previous descriptions of the five-component blend of QMP in *Apis mellifera* L. have been done by Slessor et al. (1988, 1990). The study presented here analyzes a greater number and range of queen types and permits a more complete description of QMP in *Apis mellifera*. The queen types examined here include European mated, virgin, and drone-laying queens, representing various female honey bee reproductive types, and mated Africanized queens. Africanized bees are derived from South African bees accidentally released in

South America. The rapid expansion of Africanized honey bees throughout South and Central America can be attributed to their propensity to higher swarming rates with smaller swarm sizes (Winston et al., 1983). Since QMP is important to colony cohesion and plays a role in the timing of swarming both in European and Africanized honey bees (Pettis et al., 1994; Winston et al., 1991), QMP difference may influence differences in swarming behavior between these races.

METHODS AND MATERIALS

Queen Collection and Mandibular Gland Component Analysis

Queens were immediately placed on Dry Ice upon collection, then into labeled Eppendorf tubes, and stored at −70°C prior to dissection, except where stated otherwise. Mandibular glands were extirpated, then extracted in 2 × 50 μl of methanol containing 10-undecenoic acid (0.22 mg/ml) as an internal standard. Two-microliter aliquots of the extracts were derivatized with BSTFA (Slessor et al., 1990), diluted with hexane and analyzed by splitless capillary gas chromatography on an HP5890 gas chromatograph equipped with a flame ionization detector (FID), and a 30-m DB-1 fused-silica capillary column. The FID was calibrated with standards of known concentration, and these FID responses were used to calculate the amount of each component in the mandibular gland extracts.

European Mated Queens. Mandibular gland extract data from 125 1- and 2-year-old mated queens of Simon Fraser University stock most similar to *Apis mellifera ligustica* were combined to establish a baseline mated queen mandibular gland component profile. Data from 10 queens have not been reported previously, and the remainder are from Slessor et al. (1990), Pankiw et al. (1994), and Pettis et al. (1995).

Virgin Queens. Ten virgin queens of Simon Fraser University stock most similar to *Apis mellifera ligustica* were reared until 2 weeks old and then collected in July and August 1992. Mandibular gland component analysis results from these queens were combined from seven queens from Slessor et al. (1990) for a total of 17 queens.

European Drone-Laying Queens. Twenty-two queens of mixed stock most similar to *Apis mellifera ligustica* were narcotized two times with CO_2 seven days after emergence and instrumentally inseminated with insect saline solution to induce haploid male egg laying. They then were collected from five-frame nucleus colonies at 8 weeks of age on August 21 and 22, 1991, as above. Queens were dissected from May 20 to June 6, 1992.

Africanized Mated Queens. Twenty mated Africanized queens were obtained from Mexico in 1992. Morphometric (Daly and Balling, 1978) analysis of the progeny confirmed the racial status of these queens. Queens were transported

on Dry Ice by overnight courier from Mexico and received March 3, 1992. The queens were stored at −70°C prior to dissection from June 22 to 29, 1992.

Proportions and Ratios

To develop an ontological profile of queen mandibular gland components, the results reported in Slessor et al. (1990), Pankiw et al. (1994), Pettis et al. (1995), and this study were combined. Ratios of HDA, HOB, and HVA to ODA were calculated to develop a definitive formula of a QMP queen equivalent.

Statistical Analysis

Analysis for statistical differences between European mated and all other queen types were performed using the one-tailed *t* test Satterthwaite method for unequal variances (Snedecor and Cochran, 1980). Analysis for statistical differences between European mated and all other queen types for proportion and ratio data were by the Kruskal-Wallis test (Conover, 1980). SAS was used for all statistical analyses. The level of significance throughout was at the 0.05 level, unless stated otherwise.

RESULTS

Quantities. Glands of European mated queens contained statistically greater quantities of all QMP components compared to virgin, drone-laying, and Africanized queens (Figure 1), except for a statistically similar quantity of 9-HDA in Africanized and European mated queens (Figure 1).

Proportions and Ratios. Queen mandibular gland biosynthetic capabilities appear to be ontogenetic (Figure 2); an examination of individual QMP component proportions to the total amount of QMP reveals a reproductive shift in the pheromonal "bouquet" (Figure 2). Virgin queen glands contained high proportions of ODA and lower 9-HDA proportions. Drone-laying queen ODA and 9-HDA proportions were intermediate between virgin and mated queens. Mated queen glands contained significantly greater proportions of 9-HDA compared to virgin queens (Figure 2). Africanized queen ODA proportions were significantly lower, and 9-HDA higher, compared to mated European queens.

The aromatic component (HOB and HVA) proportions changed significantly with reproductive status (Figure 2). Virgin and drone-laying queen glands contained significantly lower proportions of the aromatic components. Africanized queen gland aromatic proportions were similar to mated European queens (Figure 2).

The ratios of 9-HDA, HOB, and HVA to ODA in the glands for the various

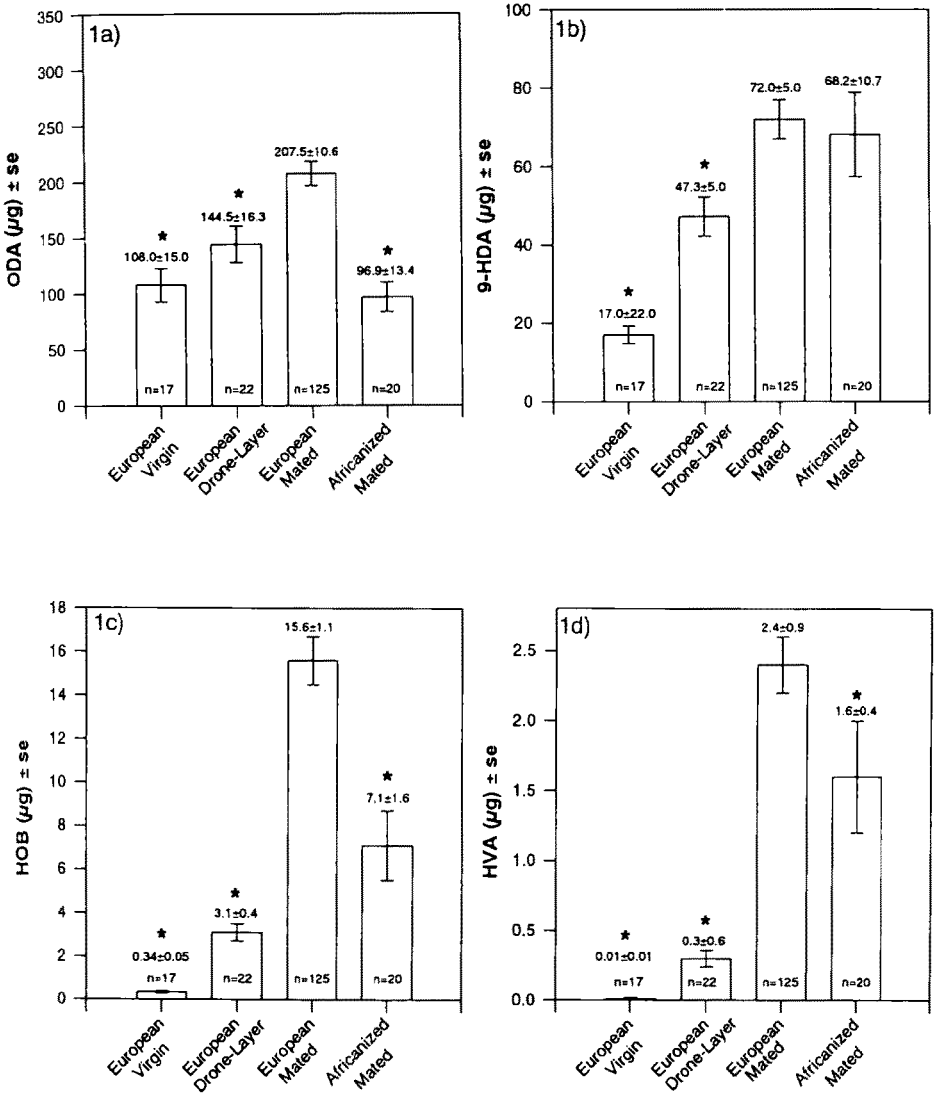

FIG. 1. Whole gland quantities of individual queen mandibular pheromone components. An asterisk above a bar indicates a statistically significant difference from the mated European queen mean at the 0.05 level; (a) ODA, 9-keto-(*E*)-2-decenoic acid; (b) 9-HDA, 9-hydroxy-(*E*)-2-decenoic acid; (c) HOB, methyl *p*-hydroxybenzoate; (d) HVA, 4-hydroxy-3-methoxyphenylethanol.

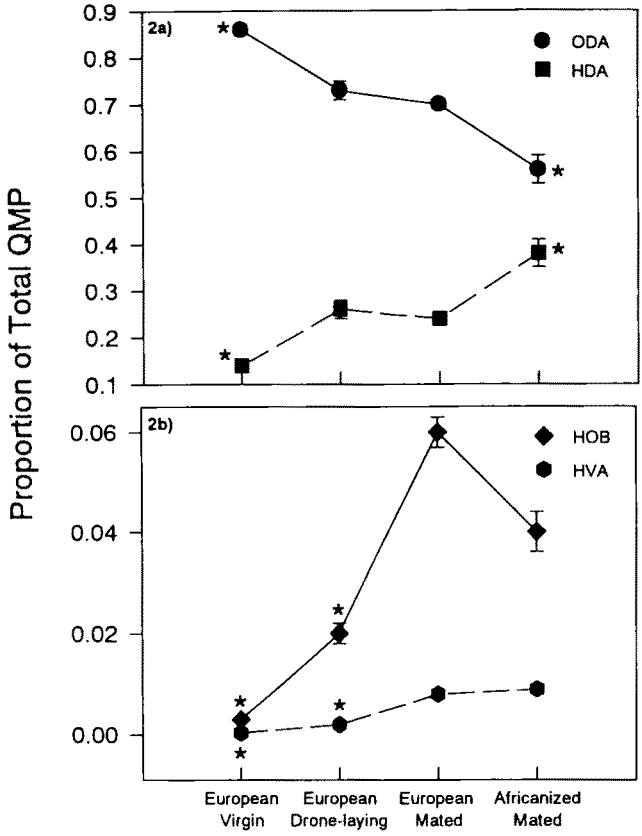

FIG. 2. Ontogenetic and reproductive profile of QMP extracted from glands. An asterisk indicates a statistically significant difference from the mated European queen mean at the 0.05 level. Invisible standard error bars are less than the size of the symbol.

queens are summarized in Table 1. The gland ratio of 9-HDA/ODA was significantly higher in Africanized than European mated queens and significantly lower in the virgin queens. The virgin and drone-laying queen glands contained significantly lower ratios of HOB/ODA, and HVA/ODA compared to the European queens, whereas Africanized queen glands contained similar ratios.

Table 2 demonstrates examples of calculated queen equivalents of glandular QMP based on the mean ODA values from Figure 1a.

Frequency Distributions. Frequency distributions of QMP component quantities of the mated European queens (Figure 3) indicated a wide range of variability in the composition of this pheromone.

TABLE 1. MEAN RATIOS OF QMP COMPONENTS TO ODA FOR VARIOUS QUEEN TYPES

Queen type	Ratios		
	9-HDA/ODA	HOB/ODA	HVA/ODA
European mated	0.40	0.09	0.01
Africanized mated	0.75[a]	0.07	0.02
European drone-laying	0.40	0.03[a]	0.002[a]
European virgin	0.20[a]	0.004[a]	0.0004[a]

[a]Significant ($P < 0.0002$) difference from mated European queen.

TABLE 2. CALCULATED VALUES FOR ONE QUEEN EQUIVALENT OF QMP

Queen type	Queen equivalent QMP components (μg)[a]			
	Mean ODA	Calculated 9-HDA	Calculated HOB	Calculated HVA
European mated	208 (200)	83 (80)	19 (20)	2 (2)
Africanized mated	97 (100)	39 (40)	7 (10)	2 (2)
European drone-layer	145 (145)	58 (60)	4 (5)	0.4 (1)
European virgin	108 (100)	22 (20)	0.8 (1)	0.7 (1)

[a]Values in parentheses are proposed "standard" queen equivalent values.

DISCUSSION

We define one queen equivalent (QE) based on the mean ratios of 9-HDA, HOB, and HVA quantities to ODA for the various queen types (Tables 1 and 2). A standard queen equivalent for the mated European queens, derived by rounding off from values in Table 2 is: 200 μg ODA, 80 μg 9-HDA, 20 μg HOB, and 2 μg HVA. Proportions of the total amount of QMP also may be used to achieve similar queen equivalent values. The primary purpose for proposing a queen equivalent definition is to reflect racial and reproductive differences, while incorporating the variation found in nature. Previously reported definitions of one queen equivalent used for synthetic formulations were: (1) 150 μg ODA, 55 μg 9-HDA, 13 μg HOB, and 1.5 μg HVA (Slessor et al., 1988), and (2) 250 μg ODA, 150 μg 9-HDA, 20 μg HOB, and 2 μg HVA (Slessor et al., 1990). These two queen equivalent values were formulated based on the most current knowledge of mean queen equivalent QMP component values at the time and fit well within the distributions reported here (Figure 3).

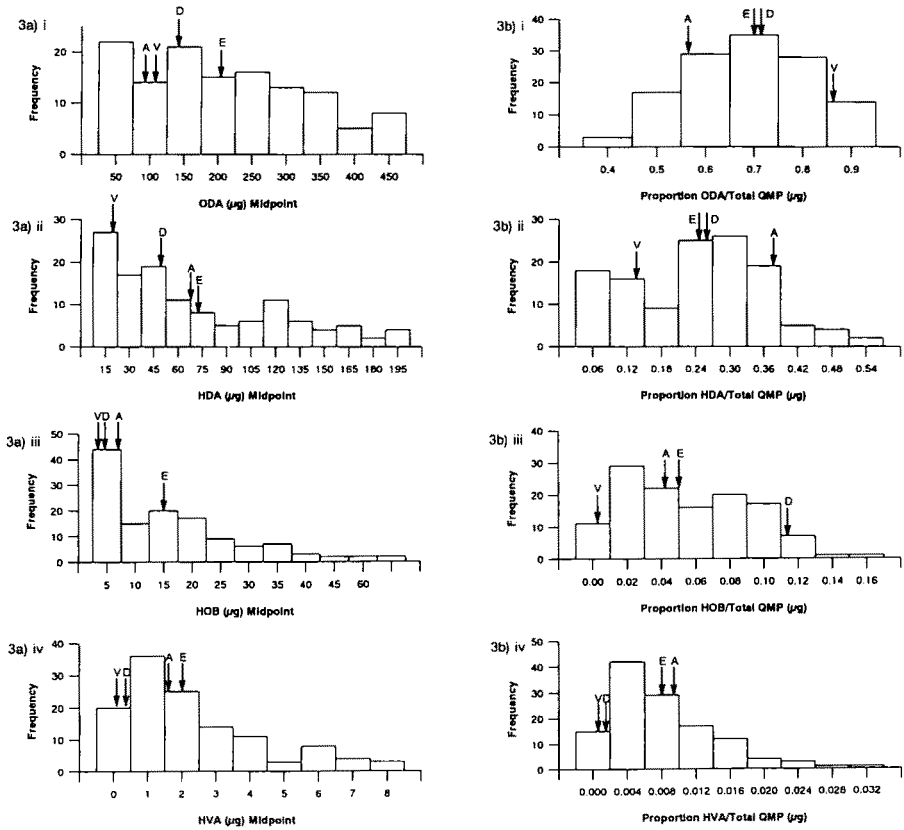

FIG. 3. Frequency distribution histograms of quantity and proportions of QMP components of 125 mated European queens: A = mean, Africanized mated queens; D = mean, European drone-laying queens; E = mean, European mated queens; V = mean, European virgin queens. Midpoint represents the median value of a range, e.g., in Figure 3a)i, a midpoint of 50 µg includes amounts of ODA greater than or equal to 0 µg and less than 75 µg ($0 \leq 50 < 75$).

The first queen equivalent definition was more reflective of mean ratios than the second (Table 1).

The comparisons between European and Africanized mated queens reveal some differences in the quantities and proportions of QMP components in these two honey bee races. However, this trait would not be reliable for classifying individual queens into a race category due to the high variability between individual queens (Figure 3). For all variables, Africanized means fall within high

frequency categories of European means, except perhaps the quantity of 9-HDA. Crewe (1982) reported differences in the proportion of mandibular gland components among *A. m. adansonii*, *A. m. mellifera*, and *A. m. capensis*, but discriminant analysis indicated that gland components were not dependable predictor variables for grouping inbred lines. Methodological differences between previously published African queen mandibular gland component quantities or percentages (Crewe, 1982; Crewe and Velthuis, 1980; Allsopp, 1988) and results reported in this study make comparisons unrealistic, but comparative studies would be of interest in the future.

Queens of either race may head colonies composed of workers of both races (Winston, 1992), and thus it appears that workers will accept racially different queen signals. Further, a European-like synthetic blend of QMP is equally effective at preventing queen rearing in colonies of either European or Africanized bees (Pettis et al., 1994). Nevertheless, the lower total quantity of QMP found in Africanized queens may contribute to the tendency for Africanized colonies to swarm with smaller parent colony sizes. The higher proportion of 9-HDA in Africanized queen glands (Figures 1 and 2) may reflect an adaptation to greater swarming activity in this race, because 9-HDA is an important component for swarm clustering behavior (Winston et al., 1982).

Unmated virgin queens not only produce significantly lower quantities of QMP components, but the proportions of components are significantly different from mated queens (Figures 1 and 2). The older (8 weeks) drone-laying queen glands contained ODA and 9-HDA proportions similar to the European mated queens, but the younger (6–14 days old) virgin queen proportions were significantly different from the mated queens. The older drone-laying queen ODA and 9-HDA proportions may reflect ontogenetic development and enzymatic capabilities of the gland. Conversely, the significantly lower proportions of the aromatic components represent a nonreproductive status (Figure 2) (Slessor et al., 1990). Virgin queens were producing a significantly different signal from the mated queens, suggesting an important biosynthetic change occurs with mating that cannot be explained by age alone, as seen by the intermediate signal produced in drone-laying queens. The essence of the different signals is a combination of quantity and proportion.

The wide variation in the quantity of QMP components suggests a generic signal indicating the individual is a queen, but there are important ontogenetic and reproductive differences in the signal as well. More will need to be known about individual QMP component biological and behavioral influences before any inferences can be made concerning the meaning of total or relative presence of any component in the blend.

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EFFECTS OF NITROGEN FERTILIZATION ON SECONDARY CHEMISTRY AND ECTOMYCORRHIZAL STATE OF SCOTS PINE SEEDLINGS AND ON GROWTH OF GREY PINE APHID

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Abstract—Effects of nitrogen availability on secondary compounds, mycorrhizal infection, and aphid growth of 1-year-old Scots pine (*Pinus sylvestris* L.) seedlings were studied during one growing season. Seedlings were fertilized with nutrient solutions containing low, optimum, and two elevated (2× and 4× optimum) levels of NH_4NO_3. At the end of growing season foliar nitrogen concentration, needle biomass, needle length, water contents of needles, root collar diameter, and number of buds increased with enhanced nitrogen availability. Addition of nitrogen did not have effect on concentrations of monoterpenes in growing needles, but in mature needles significantly decreased concentrations of some individual and total monoterpenes were detected. In growing needles the concentrations of some individual resin acids decreased, and in mature needles concentrations of some individual and total resin acids increased with increased nitrogen fertilization. Higher numbers of resin ducts were found in mature needles with nitrogen fertilization. Nitrogen fertilization decreased total phenolic concentrations in growing and mature needles of the current year, but in needles of the previous year no significant differences occurred. Mycorrhizal infection was highest at medium (optimum and 2× optimum) nitrogen fertilization levels. The relative growth rate (RGR) of grey pine aphid [*Schizolachnus pineti* (F.)] responded positively to the increase in foliar nitrogen content. However, the increase in aphid performance between optimum and the highest fertilization level was slight. This may indicate a deterring effect of resin acids on aphids. The results indicate that carbon/nutrient balance hypothesis fails to predict directly the effects of nitrogen availability on concentrations of carbon-based defensive compounds in mature foliage. Altered nitrogen supply affects allocation to secondary

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metabolites differently, depending on the developmental state of the plant and the biosynthesis pathway, cost of synthesis, and storage of compounds.

Key Words—Nitrogen, *Pinus sylvestris*, monoterpenes, resin acids, total phenolics, resin canals, plant–insect interactions, *Schizolachnus pineti*, mycorrhiza.

INTRODUCTION

Scots pine (*Pinus sylvestris* L.) is normally adapted to grow on nutrient-poor sites. An excess of nitrogen increases the growth and changes the phenology of pine seedlings (Will, 1971; Lämsä et al., 1990; Rikala and Huurinainen, 1990; Dewald et al., 1992). Pines produce significant amounts of secondary compounds: phenolic compounds and oleoresin, which mainly contains monoterpenes and resin acids. These substances may have an important role in defense responses against herbivory (Alfaro et al., 1980; Larsson et al., 1986; Schopf, 1986; Elliott and Loudon, 1987; Ross and Berisford, 1990) and pathogen attacks (Flodin and Fries, 1978; Haars et al., 1981; Stenlind and Johansson, 1987; Lieutier et al., 1991; Himejima et al., 1992). Investigations of the interactions between leaf nitrogen and carbon-based chemical defense suggest that both nitrogen and allelochemicals are important factors in determining the nutritional quality of plant tissue for herbivores (Watt et al., 1990), but the response of sucking insects to host plant quality on conifers is poorly known (Waring and Cobb, 1992).

Variations in environmental conditions can modify the quantities of secondary compounds found in plants (Gref and Tenow, 1987; Björkman et al., 1991; Kainulainen et al., 1992; Muzika, 1993). Limited availability of resources, particularly nitrogen, may determine the plant investment in secondary compounds (Bryant et al., 1983; Coley et al., 1985). Bryant et al. (1983) have proposed that the carbon/nutrient balance of individual plants strongly affects their allocation of resources to primary and secondary metabolism. They predicted that increase in availability of nitrogen to plants would increase allocation of carbon to growth, resulting in a corresponding reduction of carbon-based secondary compounds, e.g., terpenes and phenolics. However, changes in terpenoid levels have not conformed to this hypothesis as well as do changes in the levels of other carbon-based compounds, such as phenolic compounds (Bryant et al., 1987; Muzika and Pregitzer, 1992; Muzika, 1993). For example, in pine needles, concentrations of terpenoid compounds increased after nitrogen fertilization (Björkman et al., 1991; McCullough and Kulman, 1991). Terpenoid compounds are synthesized and stored in resin canals, which are specialized anatomical structures. Björkman et al. (1991) suggested that the formation of resin ducts is limited by the low availability of nitrogen.

In mycorrhizal root systems the carbon allocation and nitrogen uptake are closely related (Scheromm et al., 1990; Plassard et al., 1991). Both shortage and surplus of nitrogen are reported to affect adversely ectomycorrhiza development (Marx et al., 1977; Alexander and Fairley, 1983; Väre, 1990; Holopainen and Heinonen-Tanski, 1993). Plants with well-developed ectomycorrhiza were able to allocate more carbon to shoot growth and antiherbivore defense due to better water and nutrient uptake (Gehring and Whitham, 1994). However, carbon/nutrient balance hypothesis (Bryant et al., 1983) does not take into account the changes in nutrient uptake by mycorrhiza and costs of mycorrhiza formation.

The present study measures the responses of secondary compounds, mycorrhiza, and parasite interactions of Scots pine seedlings to different nitrogen availability regimes (low, optimal, and elevated levels). The primary objective was to find out if there is variation in concentrations of secondary compounds during shoot and needle development and if secondary chemical compounds originating from separate metabolic pathways have similar responses to nitrogen availability. A further objective of the study was to detect the possible role of increased nitrogen availability to mycorrhizal symbiosis and phloem-feeding herbivores, because these biotic interactions are often disturbed when the host plant is subjected to increased nitrogen deposition, and they may affect resource allocation patterns in host plant.

METHODS AND MATERIALS

Plant Material and Fertilizer Treatment. Scots pine (*Pinus sylvestris* L.) seedlings (seed origin: Kaskimäki, Toivakka, M29-90-0004, 1 Mk PS-508) were obtained from the forest nursery of Suonenjoki (62°38′N, 27°04′E), central Finland. Seeds were sown in spring in paper pots (PS-508: 124 cm^3) filled with *Sphagnum* peat (Vapo Tree nursery peat; mixture fertilizer 11-11-18 (N-P-K) 1 kg/m^3, dolomite lime 3 kg/m^3, total nitrogen 8 g/m^2). The plants were overwintered in an open field at the Botanical Garden of the University of Kuopio. In the beginning of January, the plants were moved into a greenhouse and kept at a temperature of 5°C for a few days. The seedlings with their peat pot were planted in sand (grain size 0.5–1.2 mm) in 0.5-liter plastic pots (diameter 10.5 cm) with bottom perforations to prevent flooding and kept in a greenhouse at 14°C for one week. After that the plants were grown in a controlled growth chamber simulating the weather conditions of central Finland in June: day/night temperature was 19/12°C, the light/dark cycle 22/2 hr and the maximum light irradiance about 300 μmol photons/m^2 sec.

At the beginning of the period of new shoot growth, the seedlings were arranged into four blocks. One block of seedlings was fertilized with a solution

(50 mg N/liter) that contained the nitrogen and cation nutrition requirement for optimal growth of Scots pine (Ingestad, 1962). Two other blocks were fertilized with nitrogen levels two (100 mg N/liter) and four times (200 mg N/liter) higher than the optimum level. Seedlings were fertilized three times a week and the fourth block of seedlings received the equivalent volume of distilled water (100 ml) at each fertilization time.

Sampling. Eight seedlings of each fertilization level were sampled when the seedlings had received nitrogen equivalent to 0, 9.8, 19.6, and 39.3 g/m^2 (0, 85, 170, and 340 mg N per pot) and needles were still growing. The second sampling was made when the growth of new shoots and needles had ceased with nitrogen concentrations equivalent to 0, 16.7, 33.5, and 67.0 g/m^2 (0, 145, 290, and 580 mg N per pot). Needles and roots of seedlings were collected for secondary chemistry, light microscopy, and mycorrhizal analysis in both samples. Secondary chemistry and light microscopy samples were collected from needles of the current year. Total phenolics were also analyzed from needles of the previous year. Roots were carefully washed and divided into two lots, which were used for mycorrhizal and total phenolic analysis, respectively. In the latter sample, total nitrogen was analyzed from current-year needles of separate seedlings, which had been used in the measurement of the aphid growth rate.

Morphological and Biomass Measurements. At both sampling dates, the height of the seedlings and the water content of the needles of the current and previous years were measured. The fresh weights of current-year shoots were measured from actively growing seedlings in the first sampling. In the latter sampling root collar diameter, needle length, number of buds, and biomass of foliage were measured. Needles and stems for biomass and water content measurements were oven-dried at 60°C for two days and weighed.

Chemical Analysis. Monoterpenes were extracted from fresh needles with *n*-hexane (Kainulainen et al., 1992). Resin acids were extracted from freeze-dried needles following the procedures of Gref and Ericsson (1985). Only abietane and pimarane diterpene resin acids were investigated. Extracts were analyzed using gas chromatography–mass spectrometry (Hewlett Packard GC type 5890, MSD type 5970) using a 25-m-long HP-5 (0.2 mm ID, 0.11 μm film thickness, Hewlett Packard) capillary column as described earlier by Kainulainen et al. (1994).

Total phenolic analysis of current-year needles were made from the same needle powder as resin acids. For total phenolic analysis previous-year needles and roots were oven-dried at 45°C for two days and milled. The samples were stored in a desiccator at 4°C. Needle powder was extracted with 80% (v/v) aqueous acetone (Kainulainen et al., 1993), and total phenolics were analyzed by the Folin-Ciocalteu technique as described by Julkunen-Tiitto (1985). Tannic acid was used as a standard and results expressed as tannic acid equivalents.

Needles for total nitrogen analysis were oven-dried at 60°C for 48 hr and milled. Total nitrogen was analyzed by the standard Kjeldahl technique (Allen, 1989).

Sample Preparation for Light Microscopy. Needle samples were immediately placed in 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.0). Pieces (about 1 mm) cut from a region 1/3 behind the tip of each needle were prefixed for 20 hr in glutaraldehyde fixative and postfixed in 1% OsO_4 solution for 6 hr at 6°C. After fixation, samples were dehydrated in a graded ethanol series and embedded in Epon LX-112. Blocks were polymerized for one day at 37°C and three days at 60°C. Sections for light microscopy (five replicates) were stained with toluidine blue and studied at magnifications of 100× or 400×. The number and diameter of resin ducts and the cross-sectional area of needles were measured.

Mycorrhizal Infection. The washed root systems of the seedlings that were used for secondary chemistry analysis were stored at −24°C until analyzed. Each root system was positioned over mesh and a total of 1 m of young long roots were randomly sampled. The mycorrhizal root tips were identified under a dissecting microscope after 15 min staining by Ponceau S (Daughtridge et al., 1986). The root systems were highly dominated by one mycorrhizal type—thin-sheathed brown mycorrhizae. Therefore, only the total number of mycorrhizae was counted, and the percentage of rootlets infected by mycorrhizal fungi was used for treatment comparisons.

Aphid Growth. Bioassay with aphids was accomplished on plants that were later used for total nitrogen analysis. Third-instar nymphs of the grey pine aphid [*Schizolachnus pineti* (F.)] reared on pine seedlings receiving optimum fertilization were placed on needles of the current year when the plants had received nitrogen at 0, 16.7, 33.5, and 67.0 g/m^2. The relative growth rate (RGR) was determined by weighing the nymphs before transfer and after three days of feeding on experimental seedlings. The nymphs were weighed individually on a Sartorius Microbalance MP3 (resolution 1 μg). RGR for nymphs was calculated with the formula: $(\ln W_2 - \ln W_1)/t$, where W_2 is final weight, W_1 is initial weight, and t is time in days between the two measurements.

Statistical Analysis. Data were analyzed with one-way analysis of variance and differences between optimal and other nitrogen treatments were compared with orthogonal contrasts (Mize and Schultz, 1985).

RESULTS

Total Nitrogen and Growth. The total nitrogen concentration of needles was significantly increased with higher nitrogen fertilization (Table 1). No sig-

TABLE 1. EFFECTS OF NITROGEN FERTILIZATION ON GROWTH, BIOMASS PRODUCTION, AND TOTAL NITROGEN CONCENTRATION OF PINE SEEDLINGS[a]

	Nitrogen fertilization level (g/m^2)				ANOVA		*P* for contrasts		
	0	16.7	33.5	67.0	*F* value	*P*	16.7 vs 0	16.7 vs 33.5	16.7 vs 67.0
Total height (cm)	15.0 (17)	16.5 (18)	16.3 (17)	16.6 (16)	1.86	0.145	0.050	NS	NS
Root collar diameter (mm)	3.1 (17)	4.4 (18)	4.9 (17)	5.1 (16)	36.33	<0.001	<0.001	0.028	0.003
Needle length (cm)	2.9 (17)	9.3 (18)	11.0 (17)	11.0 (16)	112.22	<0.001	<0.001	0.002	0.001
Number of buds	3.4 (17)	4.8 (18)	5.5 (17)	5.7 (16)	10.42	<0.001	0.002	NS	NS
Biomass of current year needles (g dw)	0.65 (9)	2.85 (10)	3.31 (9)	3.82 (8)	34.80	<0.001	<0.001	NS	0.007
Needle moisture (%) current year	56.2 (17)	68.3 (18)	69.4 (17)	70.7 (16)	67.30	<0.001	<0.001	NS	0.041
Needle moisture (%) previous year	49.1 (16)	56.0 (17)	55.4 (17)	55.8 (16)	10.79	0.001	<0.001	NS	NS
Foliar nitrogen (mg/g dw)	3.8 (8)	11.5 (8)	15.5 (8)	18.9 (8)	112.43	<0.001	<0.001	<0.001	<0.001

[a]Number of seedlings in parentheses. *F* and *P* values are from one-way analysis of variance. Differences from optimal nitrogen level (16.7 g/m^2) are compared with orthogonal contrasts. NS = not significant.

nificant differences were found in the height of the seedlings, but the appearance of seedlings was quite different among nitrogen fertilization levels. Needles were much longer and relatively easy to detach from the stem at the highest nitrogen fertilization levels compared to lower nitrogen levels. Furthermore, the seedlings with nitrogen fertilization had darker green needles than the seedlings that received only water. The biomass of needles increased linearly with enhanced nitrogen fertilization. The root collar diameter and the number of buds also increased with higher nitrogen availability. The water content of current- and previous-year needles increased with nitrogen fertilization.

Secondary Compounds. The major monoterpenes were α-pinene, 3-carene, camphene, and myrcene, which cumulatively accounted for 75–90% of total monoterpenes. Concentrations of monoterpenes in growing needles were not significantly affected by nitrogen fertilization (Table 2). However, in mature needles concentrations of tricyclene, α-pinene, camphene, myrcene, and total monoterpenes were significantly affected by nitrogen fertilization (Table 3). Concentrations of these monoterpenes were highest at the lowest nitrogen fertilization level.

Of the resin acids studied, abietic acid was the most common. In the growing needles the concentrations of levopimaric, palustric, and neoabietic acids were significantly reduced by enhanced nitrogen fertilization (Table 2). By contrast, in the mature needles the concentrations of isopimaric, palustric, neoabietic, and total resin acids were significantly increased with addition of nitrogen (Table 3).

The total phenolic concentrations of growing and mature needles of the current year were significantly affected by nitrogen fertilization (Tables 2 and 3). In mature needles total phenolic concentration at the lowest nitrogen fertilization level was significantly higher than in the optimum level, but higher fertilization levels did not reduce total phenolic concentrations. Nitrogen availability did not significantly affect the total phenolic concentrations in the previous-year needles.

Total phenolic concentration of roots was not significantly affected by nitrogen fertilization in the first sample (Table 2), but in the second sample concentrations were significantly lower at the 0 g/m^2 level than at the optimum fertilization level (Table 3).

Needle Anatomy. In growing needles the number of resin ducts was significantly higher at the highest nitrogen fertilization level than at the optimal nitrogen fertilization level (Figure 1). By contrast, in mature needles nitrogen fertilization increased the number ($r^2 = 0.287$, $P = 0.018$) and total cross-sectional area ($r^2 = 0.263$, $P = 0.025$) of resin ducts, but did not affect the diameter of resin ducts. There was a significant positive correlation between the cross-sectional area of growing ($r^2 = 0.335$, $P = 0.009$) and mature ($r^2 = 0.472$, $P = 0.001$) needles and nitrogen fertilization level.

TABLE 2. MEAN CONCENTRATIONS OF MONOTERPENES, RESIN ACIDS, AND TOTAL PHENOLICS IN GROWING SCOTS PINE NEEDLES (N = 8) AT DIFFERENT FERTILIZATION LEVELS[a]

	Nitrogen fertilization level (g/m^2)				ANOVA		P for contrasts		
	0	9.8	19.6	39.3	$F_{3,27}$	p	9.8 vs 0	9.8 vs 19.6	9.8 vs 39.3
Monoterpenes (μg/g fw)									
Tricyclene	8	7	6	5	2.76	0.061	NS	NS	NS
α-Pinene	150	205	141	174	1.62	0.205	NS	NS	NS
Camphene	51	45	37	30	2.03	0.132	NS	NS	NS
β-Pinene + sabinene	24	20	22	16	1.63	0.206	NS	NS	NS
Myrcene	54	46	48	52	0.30	0.828	NS	NS	NS
3-Carene	90	72	147	86	1.71	0.188	NS	0.045	NS
Limonene	28	24	19	15	1.53	0.229	NS	NS	NS
Bornylacetate	17	11	11	9	1.03	0.393	NS	NS	NS
Total	424	432	435	390	0.29	0.833	NS	NS	NS
Resin acids (μg/g dw)									
Isopimaric acid	614	529	527	463	0.38	0.765	NS	NS	NS
Levopimaric acid	100	24	44	29	4.56	0.010	0.003	NS	NS
Palustric acid	221	205	51	17	7.58	<0.001	NS	0.008	0.002
Dehydroabietic acid	668	588	597	567	0.12	0.946	NS	NS	NS
Abietic acid	1584	1337	1463	1070	0.92	0.445	NS	NS	NS
Neoabietic acid	661	835	281	95	5.35	0.005	NS	0.012	0.001
Total	3849	3519	2965	2242	1.49	0.240	NS	NS	NS
Total phenolics (mg/g dw)									
Needles (current)	59.4	53.0	38.4	44.0	4.46	0.011	NS	0.023	NS
Needles (previous)	71.6	84.2	82.7	84.3	1.70	0.189	NS	NS	NS
Roots	32.1	35.0	32.9	34.3	0.33	0.802	NS	NS	NS

[a] F- and P values are from one-way analysis of variance. Differences from optimal nitrogen level (16.7 g/m^2) are compared with orthogonal contrasts. NS = not significant.

[b] β-Pinene and sabinene were not separated from each other on HP-5 column.

TABLE 3. MEAN CONCENTRATIONS OF MONOTERPENES, RESIN ACIDS, AND TOTAL PHENOLICS IN MATURE SCOTS PINE NEEDLES (N = 8) AT DIFFERENT FERTILIZATION LEVELS[a]

	Nitrogen fertilization level (g/m^2)				ANOVA		P for contrasts		
	0	16.7	33.5	67.0	$F_{(3,27)}$	P	16.7 vs 0	16.7 vs 33.5	16.7 vs 67.0
Monoterpenes (μg/g fw)									
Tricyclene	17	9	4	7	10.83	<0.001	0.002	NS	NS
α-Pinene	310	191	174	230	3.33	0.034	0.017	NS	NS
Camphene	101	57	28	48	9.18	<0.001	0.005	0.050	NS
β-Pinene + sabinene[b]	38	26	18	50	2.38	0.091	NS	NS	NS
Myrcene	85	56	36	65	3.84	0.020	NS	NS	NS
3-Carene	78	87	96	74	0.11	0.952	NS	NS	NS
Limonene	57	26	21	41	2.49	0.081	0.040	NS	NS
Bornylacetate	58	23	26	46	1.38	0.270	NS	NS	NS
Total	747	476	406	563	5.28	0.005	0.005	NS	NS
Resin acids (μg/g dw)									
Isopimaric acid	491[c]	429	652	632	3.11	0.043	NS	0.016	NS
Levopimaric acid	79[c]	21	24	68	2.07	0.127	NS	NS	NS
Palustric acid	76[c]	192	418	494	27.16	<0.001	0.039	<0.001	NS
Dehydroabietic acid	520[c]	468	415	382	0.52	0.670	NS	NS	NS
Abietic acid	1008[c]	899	1176	1122	0.66	0.584	NS	NS	NS
Neoabietic acid	152[c]	563	1062	1129	14.75	<0.001	0.002	0.005	0.002
Total	2349[c]	2572	3749	3827	4.64	0.010	NS	0.021	0.018
Total phenolics (mg/g dw)									
Needles (current)	69.3[c]	44.2	47.0	47.5	18.65	<0.001	<0.001	NS	NS
Needles (previous)	85.0	91.2	97.7	91.4	1.58	0.216	NS	NS	NS
Roots	34.6	46.7	53.4	43.3	8.44	<0.001	0.003	NS	NS

[a] F and P values are from one-way analysis of variance. Differences from optimal nitrogen level (16.7 g/m^2) are compared with orthogonal contrasts. NS = not significant.
[b] β-Pinene and sabinene were not separated from each other on HP-5 column.
[c] Seven samples.

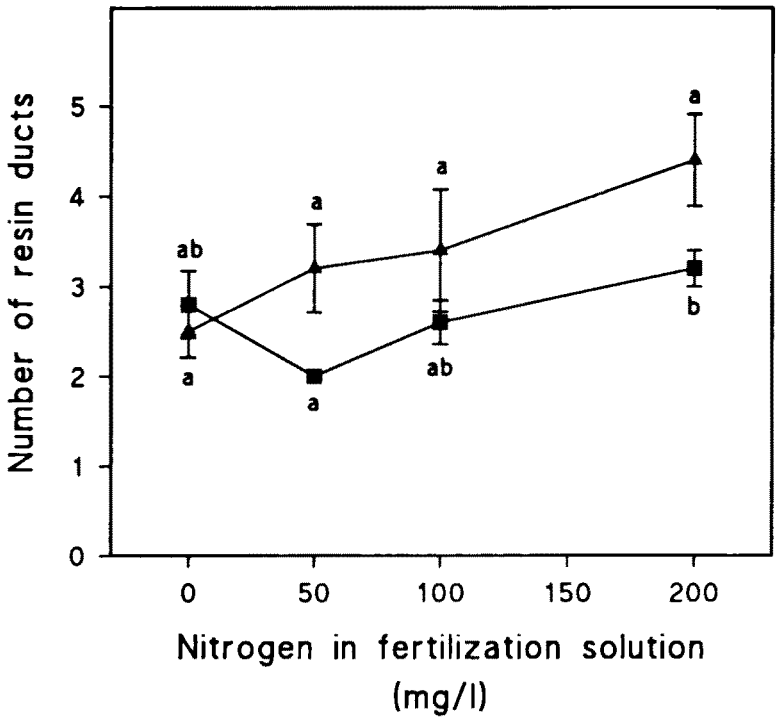

FIG. 1. Number of resin ducts at four different nitrogen fertilization levels in growing (■) and mature (▲) needles. Each value is the mean (±SE) of five samples. Mean values within a same line with the same letter are not significantly different at 0.05 level (Tukey's multiple range test).

Mycorrhizal Infection. Mycorrhizal infection responded similarly to fertilization at both sampling times (Figure 2). Both the lowest and highest nitrogen availabilities resulted in lower mycorrhizal infection levels compared to the medium fertilization levels. At the first sampling, the maximal infection was achieved by higher nitrogen fertilization than in the second sampling. Total phenolic concentration in roots did not correlate with mycorrhizal infection.

Aphid Growth. RGR of *S. pineti* nymphs was significantly ($F_{3,21} = 3.93$, $P = 0.023$) affected by nitrogen fertilization, being significantly lower ($P = 0.013$) at nitrogen fertilization of 0 g/m^2 than at the optimum level (16.7 g/m^2). RGR of nymphs was positively and nonlinearly correlated with foliar nitrogen concentration of pine seedlings (Figure 3). Secondary metabolites were not analyzed from the same seedlings that were used for bioassay. When the ratio of total nitrogen to total resin acids was calculated using treatment means, a

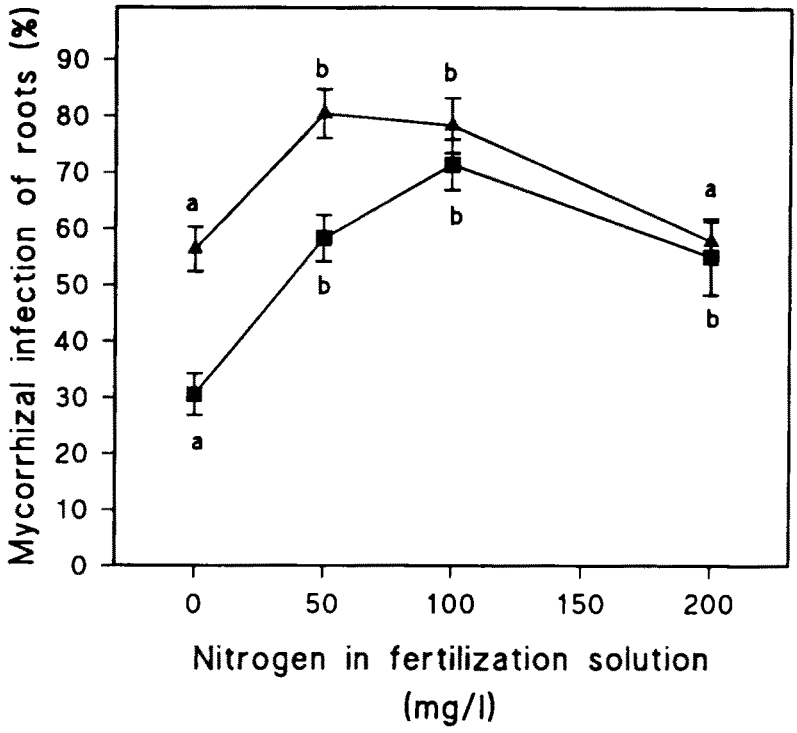

FIG. 2. Mycorrhizal infection of roots at four different nitrogen fertilization levels, in the first sampling when needles were growing (■) and the second sampling when needles were mature (▲). Each value is the mean (±SE) of eight plants. Mean values of the same line with the same letter are not significantly different at 0.05 level (Tukey's multiple range test).

significant linear positive correlation with the mean RGR of *S. pineti* nymphs per treatment was found (Figure 4). Concentrations of total nitrogen, total phenolics, total monoterpenes, and total resin acids and the nitrogen/phenolics and nitrogen/total monoterpenes ratios did not correlate with aphid RGR when treatment means were used in analyses.

DISCUSSION

Plant Growth Responses. The most distinctive growth responses of pine seedlings to increasing nitrogen availability were increases in needle length and

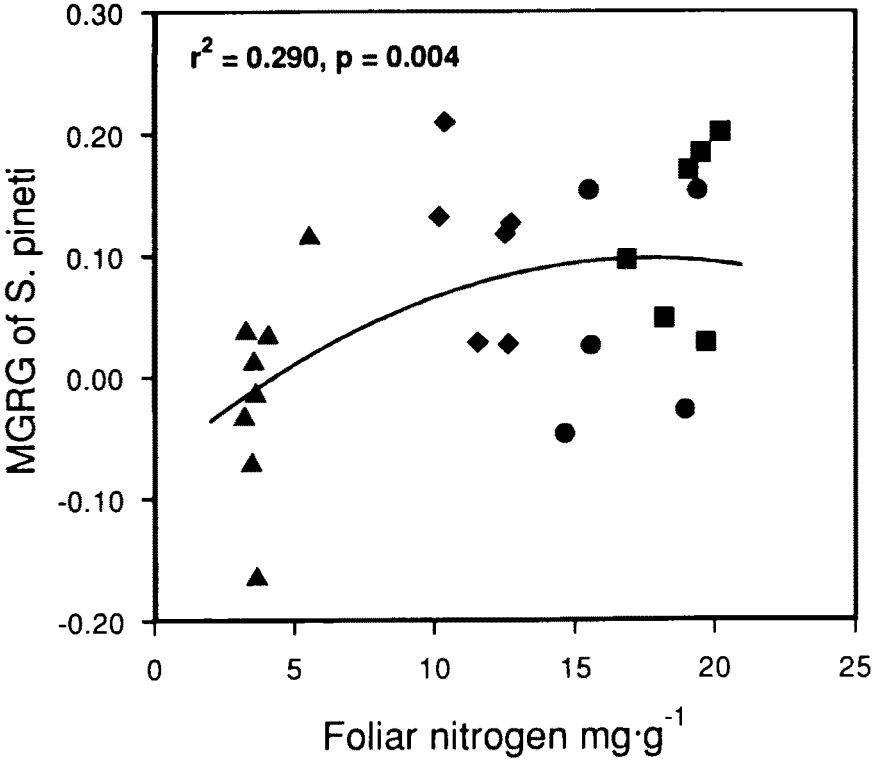

FIG. 3. Relationship between the relative growth rate of nymphs of the grey pine aphid and foliar nitrogen concentration of pine seedlings grown at four different nitrogen fertilization levels: ▲ = 0 g/m², ♦ = 16.7 g/m², ● = 33.5 g/m², and ■ = 67.0 g/m².

foliar biomass. The lack of a height growth response to nitrogen availability is explained by the fixed height growth, which is determined in Scots pine in the previous growing season during bud formation (Lanner, 1976; Kozlowski et al., 1991; Rikala and Huurinainen, 1990; Rikala, 1992). Growth allocation to photosynthesizing needle biomass reflects greater productivity and enhanced competitive ability of seedlings in response to light in a resource-rich environment (e.g., Herms and Mattson, 1992; Van der Werf et al., 1993).

Secondary Chemistry. In the present study concentrations of resin acids in mature needles increased with elevated nitrogen availability, contrary to the carbon/nutrient balance hypothesis (Bryant et al., 1983). By contrast, concentrations of foliar monoterpenes and total phenolics decreased with elevated nitro-

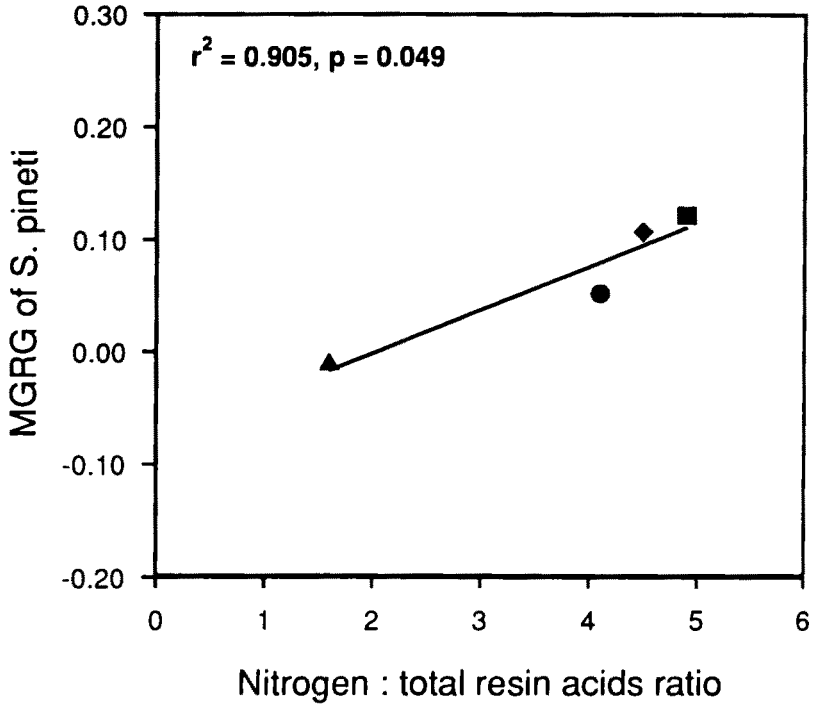

FIG. 4. Relationhip between the relative growth rate of nymphs of the grey pine aphid and foliar nitrogen/total resin acids ratio of pine seedlings grown at four different nitrogen fertilization levels: ▲ = 0 g/m^2, ♦ = 16.7 g/m^2, ● = 33.5 g/m^2, and ■ = 67.0 g/m^2 using treatment means.

gen availability, as expected by the carbon/nutrient balance hypothesis. Björkman et al. (1991) found that nitrogen fertilization resulted in higher levels of resin acids. In woody plants low nutrient availability has been observed to increase (McCullough and Kulman, 1991) or to have no effects (Thorin and Nommik, 1974; Muzika, 1993) on concentrations of monoterpenes, but in terpenoid-bearing herbs low nutrient availability stimulated monoterpene formation (Mihaliak and Lincoln, 1985; Mihaliak et al., 1987). The decreased concentrations of foliage phenolic compounds following nitrogen fertilization agree with earlier studies (Bryant et al., 1987; Muzika and Pregitzer, 1992; Hartley et al., 1995).

Terpenoid compounds, as monoterpenes and resin acids, are formed by the mevalonic acid pathway and phenolic compounds by the shikimic acid pathway. Of the major class of secondary compounds, terpenoids are among the most expensive to produce per gram (Gershenzon, 1994b). The costliest terpenoids

to produce are the ones with the lowest proportions of oxygen, e.g., monoterpenes are more expensive to produce per gram than resin acids (Gershenzon, 1994b). Overall, in Scots pine needles only a minor part of the carbon used in secondary compounds is contained in monoterpenes, while resin acids constitute a significant part of the carbon-based defense (Gref, 1982). In contrast, phenolic compounds are not especially expensive to produce (Gershenzon, 1994b), and they are stored in simple vacuoles. Terpenoid storage in complex secretory structures such as resin ducts in *Pinus* species (Lewinsohn et al., 1991) appears to impose significant costs on plants (Gershenzon, 1994b). The carbon–nutrient balance hypothesis fails to take into account the formation of multicellular storage structures needed for terpenoid synthesis and storage. The formation of these structures requires adequate supplies of nutrients, such as nitrogen, and thus terpenoid accumulation may proceed at only a limited rate under nutrient-poor conditions (Gershenzon, 1994a).

Björkman et al. (1991) observed higher resin acid concentrations in mature needles of fertilized pine seedlings as a result of the increased number and size of resin ducts. Also in the present study, the number of resin ducts and the concentrations of resin acids in mature needles were increased by fertilization. However, in growing needles the number of resin ducts was not correlated with nitrogen fertilization, and the concentrations of resin acids decreased with nitrogen fertilization. This result is consistent with our previous study of 9-week-old Scots pine seedlings, where concentrations of some individual and total resin acids in shoots decreased significantly with increasing nitrogen availability (Holopainen et al., 1995). Tobolski and Zinkel (1982) observed a large fluctuation of some resin acids during maturation of needles. It is possible that, in developing needles, if nitrogen does not strongly limit formation and synthesis of resin ducts, the excess of carbon is synthesized into resin acids. However, in mature needles the anatomy of resin canals is fully developed and the low number and size of resin ducts can limit synthesis and accumulation of resin acids at low nitrogen availability levels.

Both monoterpene (Hiltunen et al., 1975; Yazdani et al., 1982) and resin acid composition are genetically determined (Gref, 1982), but, obviously, environmental conditions can have rather strong effects on the concentrations of some compounds. Nitrogen availability had effects only on the concentration of some individual secondary compounds, while others remained unaffected. This has already been observed in many studies (Muzika et al., 1989; McCullough and Kulman, 1991; Reichardt et al., 1991; Muzika and Pregitzer, 1992; Horner et al., 1993; Muzika, 1993). In the present study concentrations of some individual resin acids—levopimaric, palustric and neoabietic acids—were affected by nitrogen fertilization, which was also seen in our previous study with 9-week-old pine seedlings (Holopainen et al., 1995). It is known that individual enzymes can yield mixtures of secondary compounds, e.g., monoterpenes and

resin acids (Gershenzon, 1994a). Probably some enzymes are more affected by environmental factors than others.

In the present study lower needle biomass was observed in seedlings with high phenolic production. In many studies, nitrogen is negatively correlated with foliage phenol concentrations (Haukioja et al., 1985; Dustin and Cooper-Driver, 1992). It has also been observed that concentrations of phenolic compounds (Ross and Berisford, 1990; Sunnerheim-Sjöberg and Hämäläinen, 1992) are inversely related to tree growth. Phillips and Henshaw (1977) observed that nitrogen inhibits accumulation of phenolics in plant cell culture, which provides evidence of the general inverse relation between nitrogen, enhanced growth, and phenolic production.

We did not find a significant correlation between mycorrhizal infection and total phenolic concentration of roots. Structural studies of roots have shown that during the mycorrhizal development, the penetrating fungus induces the formation of a tannic layer at the uppermost parts of cortex layer (Harley and Smith, 1983; Kottke and Oberwinkler, 1986), and in some other studies roots of mycorrhizal seedlings have contained significantly higher amounts of total soluble phenols than nonmycorrhizal ones (Krupa and Fries, 1971; Chakravarty and Hwang, 1991).

Symbiont and Parasite Interactions. This study indicated that ectomycorrhiza formation is retarded by both very low and high nitrogen availability, as also seen in earlier studies (Väre, 1990; Wallander and Nylund, 1991; Holopainen and Heinonen-Tanski, 1993). At the same time, aphid performance was poor at low nitrogen availability, but did not differ significantly between optimum and high nitrogen availabilities. This suggests that aphid performance is not directly related to the state of ectomycorrhizal infection. However, for some shoot parasites, such as dodder (Sanders et al. 1993), the role of mycorrhizal infection in roots can be crucial.

The interaction between mycorrhizal fungi and foliar-feeding insects is complex and very little is known of the significance of ectomycorrhizae on above-ground herbivores (Jones and Last, 1991; Gehring and Whitham, 1994). On arbuscular mycorrhizal plants aphids exhibit improved growth and reproduction compared to nonmycorrhizal plants (Gange and West, 1994). On trees, free amino acids often determine aphid performance (Kidd et al., 1990). One explanation of the lack of linear aphid growth response between optimum and higher nitrogen availabilities could be the smaller proportion of free amino acids of total nitrogen in seedlings with poor mycorrhizae at the highest nitrogen availability level, because ectomycorrhizal infection could increase the levels of free amino acids in small pine seedlings (Finlay et al., 1988; Näsholm et al., 1991). Well-developed mycorrhizae are known to increase nitrogen uptake significantly by roots (Harley and Smith, 1983), and nitrogen is translocated from the mycorrhizal roots to shoots primarily in the form of amino acids (Finlay et

al., 1988). In addition, increased nitrogen availability per se can change the balance of individual free amino acids in Scots pine seedlings (Gezelius and Näsholm, 1993) that can be harmful for *S. pineti* performance (Kidd et al., 1990).

The growth rates of *S. pineti* nymphs on new needles of nonfertilized pine seedlings was near or below zero, indicating that the needles were poorer food than needles of fertilized seedlings. Low levels of needle nitrogen and moisture or low nitrogen/total resin acid ratio, but with higher levels of total phenolics could explain this. Direct contact with oleoresin has been shown to be a primary factor in insect resistance of trees (Hanover, 1975), but the role of secondary compounds of host plant for aphid growth is poorly known (Pickett et al., 1992). Kidd et al. (1990) found that needles previously fed on by *S. pineti* are poorer food for nymphs than undamaged needles, as they have higher numbers of phenolic compounds, while total phenolics can induce phagostimulation to some stem-feeding aphid species. In field studies (Holopainen et al., 1992) there was no relationship between *S. pineti* population peaks and the foliar levels of monoterpenes and resin acids, suggesting that this aphid avoids penetrating their stylets into resin ducts. However, the present results, indicating a positive correlation in the nitrogen/total resin acids ratio, suggest that resin acids can deter aphid performance.

CONCLUSIONS

The results indicate that nitrogen availability affects allocation of carbon between growth and defense and between different pathways in the synthesis of secondary compounds. Poor nitrogen availability results in accumulation of phenolics but, contrary to the carbon/nutrient balance hypothesis, limits the concentration of carbon-based resin acids that are formed and stored in complicated multicellular structures. Reduced formation of mycorrhizae at high nitrogen fertilization levels decreases the carbon sink in roots and may partly promote carbon allocation to the formation of foliar resin acids. This can lead to decreased or increased resistance against plant parasites depending on the significance of the nitrogen/allelochemicals ratio in specific host–parasite associations. However, only controlled laboratory experiments with different fungal species can verify the actual role of ectomycorrhiza on secondary metabolism of conifers and resistance to insect herbivores.

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HOST-LOCATION KAIROMONE FROM *Periplaneta americana* (L.) FOR PARASITOID *Aprostocetus hagenowii* (Ratzeburg)[1]

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Abstract—Chemically mediated host location in the eulophid parasitoid *Aprostocetus hagenowii* (Ratzeburg) was investigated. In Y-tube bioassays 77.6% of female parasitoids responded to a *Periplaneta americana* (L.) ootheca; parasitoids did not respond to air with no volatile stimuli. Frass from adult cockroaches was as attractive as an ootheca. Bioassay of one ootheca equivalent of five lipid fractions (eluted with hexane and 1, 5, 10, and 30% ether in hexane) from silica gel column chromatography indicated that the active component was a hydrocarbon. Further separation and bioassay of oothecal hydrocarbons by $AgNO_3$-impregnated silica gel column chromatography indicated that the biological activity was in one fraction. Gas chromatographic (GC) analysis of this fraction revealed a single peak; this peak was identified by researchers in 1963, 1969, and 1972 as (Z,Z)-6,9-heptacosadiene. Qualitative and quantitative GC analyses of total hydrocarbons from oothecae, frass, and adult females were essentially identical; 6,9-heptacosadiene was the dominant hydrocarbon from each source. The alkadiene was 37 times more abundant in frass than on the ootheca. The volatilization of the alkadiene from oothecae was demonstrated by aeration and trapping on Super Q adsorbent. The current study is the first evidence for biological activ-

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ity of (*Z*,*Z*)-6,9-heptacosadiene, a major hydrocarbon component on adult female American cockroaches, on their oothecae, and in their frass.

Key Words—Biological control, cuticular hydrocarbon, attractant, ootheca, 6,9-heptacosadiene, Blattidae, frass, Insecta, *Periplaneta americana*, *Aprostocetus hagenowii*, kairomone.

INTRODUCTION

Kairomone-mediated host location has been well documented. Eggs (Leonard et al., 1987), pupae (Cardé and Lee, 1989), larvae (Vinson et al., 1975), wing scales (Jones et al., 1973; Shu et al., 1990), frass (Jones et al., 1971; Nemoto et al., 1987a,b; Auger et al., 1989; Fukushima et al., 1989), and mandibular gland secretions (Mudd, 1981; Mudd and Corbet, 1982; Mudd et al., 1984) contain compounds that elicit host-finding and host-acceptance behaviors from parasitoids. *Aprostocetus hagenowii* (Ratzeburg), a eulophid parasitoid of oothecae of cockroaches in the family Blattidae, exhibited typical host-recognition behaviors (drumming, tapping, drilling) on glass beads treated with calcium oxalate from colleterial glands and mucopolysaccharides from salivary glands of *Periplaneta americana* (L.) (Vinson and Piper, 1986). *Comperia merceti* (Compere) used the ootheca-fixing cement from the cockroach *Supella longipalpa* as a host-acceptance kairomone (Van Driesche and Hulbert, 1984).

The biology of *A. hagenowii* has been well documented (Roth and Willis, 1954, 1960; Cameron, 1955; Edmunds, 1955; Narasimham, 1984). Its preferred host is an ootheca of *P. americana*, but it will also parasitize oothecae of *P. australasiae* (F.), *P. fuliginosa* (Serville), *P. japonica* Karny, *P. brunnea* Burmeister, *Blatta orientalis* L., *Eurycotis floridana* (Walker), and *E. biolleyi* Rehn. Upon emergence from the ootheca, parasitoid siblings mate and females can immediately oviposit. Developmental time (i.e., oviposition to adult emergence) is approximately 30 days at 30°C; the sex ratio of the resultant clutch is strongly female-biased (86%). The broad host range, fecundity, and life cycle of *A. hagenowii* make it an excellent candidate for the biological control of Blattidae. In this study, we investigated a host-location kairomone for *A. hagenowii* from *P. americana* oothecae.

METHODS AND MATERIALS

Parasitoid. A. hagenowii was colonized in *P. americana* oothecae in the spring of 1993 from 10–20 adult female parasitoids collected at the Swine Research Unit on the University of Florida campus in Gainesville, Florida. A sample of parasitoids was sent to the U.S. National Museum, Washington, D.C., and identified by Dr. M. E. Schauff.

Oothecae were placed individually into 18-ml snap-cap vials (Thornton Plastics, Salt Lake City, Utah) each containing a single 2- or 3-day-old female parasitoid that had emerged from laboratory- and field-stung, sentinel oothecae placed in treehole microhabitats, a primary cockroach harborage in Florida. The vials were stored in plastic boxes (20 × 32 × 10 cm) and held at 27–30°C, in natural light (i.e., by a window) and humidity. Each day the vials containing newly emerged progeny were placed, with snap-caps intact, into a Plexiglas cage (30 × 30 × 30 cm) (Hagenbuch et al., 1988) and held for 24 hr to ensure sibling mating. After 24 hr the snap cap was removed from each vial, allowing parasitoids to mix. Water and food were provided from water and honey–water soaked cotton balls.

A. hagenowii used in this study were 1-day-old (29 ± 11 hr), unfed females that had emerged from three to five laboratory-stung *P. americana* oothecae. The day preceding bioassay parasitoids were released together into a clean Plexiglas cage (Hagenbuch et al., 1988) different from those cages used for parasitoid colony maintenance. The oothecae from which the parasitoids had emerged were removed from the cage to avoid possible habituation to the kairomone.

Oothecae. P. americana oothecae were collected in ootheca production units according to the method of Hagenbuch et al. (1988). Adult cockroaches were obtained as needed from a sewage treatment plant on the University of Florida campus (see Cornwell, 1968, Figure 115). One hundred to 300 mixed-sex adult cockroaches were placed in each tub and provided water from a chick waterer, seven polyvinylchloride harborages (5 cm ID × 12 cm long), and laboratory rat chow (23% crude protein; Purina, #5001, St. Louis, Missouri). Dead cockroaches were replaced, water and food replenished, and oothecae collected weekly.

Olfactometer. A Y-tube olfactometer similar to the one described by Vander Meer et al. (1988) was used in this study. The insect trap was created by a 24/40 ground glass joint. Breathable-quality compressed air was humidified (approximately 75% relative humidity) by passge over 100–200 ml of saturated NaCl solution before being regulated through two flowmeters (65 mm variable area, stainless steel float, 333 ml/min maximum airflow; Cole Parmer, Niles, Illinois) mounted to the inside back wall of a white, wood box (58 cm high × 74 cm wide × 69 cm deep) with an open front and top. The olfactometer was placed on the center floor; a space (3 cm high × 74 cm wide) between the floor and the bottom of the back wall allowed each flowmeter outlet tube to connect to each lateral arm of the olfactometer. All the airflow tubes were corrugated Teflon FEP tubing (Cole Parmer).

A chamber, constructed from the top half of a 50-ml Nalgene bottle (3 cm ID, 1 cm ID at the mouth), was built to allow the unprovoked, natural migration of parasitoids into the common arm of the olfactometer during a bioassay. Into

the bottom of the chamber was a fitted stainless steel screen (150 mesh; Small Parts Company, Miami, Florida) to prevent parasitoids from escaping and to allow the free flow of air from the common arm. Parasitoids were transferred from the Plexiglas holding cage into the chamber through its mouth, which was then fitted to the end of the common arm.

Ootheca and Frass Bioassays. All bioassays were conducted between 09:00 and 17:00 hr at 28–31°C. A single, clean (i.e., without attached detritus), < 10-day-old ootheca was placed distally in a lateral arm of the olfactometer. Ten parasitoids were then placed into the common arm of the olfactometer, and after 10 min the number that (1) moved into the olfactometer trap containing the ootheca, (2) moved into the olfactometer trap containing air only, and (3) failed to enter either trap was recorded. The procedure was repeated but with the ootheca in the other lateral arm of the olfactometer. Thus, a replicate was the response of 20 parasitoids. Each bioassay was conducted with a different cohort of 10 parasitoids. After each bioassay, the olfactometer was disassembled, rinsed with acetone, and fan-dried for 3–5 min. The bioassay was conducted at airflow rates of 30, 60, 120, and 240 ml/min/arm of the Y tube. Finally, the response of parasitoids to air (i.e., no ootheca in either lateral arm) was investigated at the highest airflow rate of 240 ml/min/olfactometer arm.

The bioassay apparatus was used to test 50, 100, and 200 mg samples (N = 2, 3, and 2 replicates, respectively) of 1- to 5-day-old frass from mixed sex adult and large nymphal *P. americana* for its attractiveness to *A. hagenowii*. All frass bioassays were conducted at the highest airflow rate of 240 ml/min/arm.

Isolation of Kairomone from Oothecae. Thirty < 10-day-old oothecae were sequentially immersed in hexane (99.9%; Baxter, Muskegon, Michigan) for 10, 5, 2, and 2 min to remove cuticular lipids. The washes were combined, reduced in volume under a gentle stream of nitrogen and chromatographed on a silica gel column (5 cm) (70–230 mesh; Sigma, St. Louis, Missouri) in a Pasteur pipet. Fractions were collected by gravity elution with sequential 6-ml aliquots of hexane, followed by 1, 5, 10, and 30% anhydrous ether (99.9%; Aldrich, Madison, Wisconsin) in hexane (Carroll, 1961). Each fraction was dried under nitrogen and immediately (< 1 min) reconstituted in hexane to provide one ootheca equivalent (OE) per 10 μl. For each fraction, 1 OE was pipetted onto a paper wick (3 × 14 mm; Northfork, Tumwater, Washington) and bioassayed at the highest airflow rate (240 ml/min/arm); a paper wick control wetted with 10 μl hexane was placed in the opposite lateral arm. A replicate was the response of 20 parasitoids (i.e., 10 parasitoids bioassayed for 1 OE in each lateral arm). Each replicate was expressed as a percentage of the response to an ootheca on the day fractions were bioassayed (i.e., on the same day, the number of parasitoids responding to a fraction divided by the number responding to an ootheca, typically 7–9 of 10).

Cuticular oothecal lipids were removed as described previously, and total hydrocarbons collected by column elution with hexane (Brill and Bertsch, 1985). Total hydrocarbons were then reduced in volume and chromatographed on a column (3 cm) of 10% $AgNO_3$-impregnated silica gel (200+ mesh; Aldrich). Fractions were collected by gravity elution with hexane followed by 2% and 10% ether in hexane (Carlson and Service, 1980). The resulting fractions were passed through a 1-cm column of silica gel and eluted with 2 ml of hexane to remove residual silver ions. Fractions were dried, reconstituted in hexane, and bioassayed.

Collection of Frass and Adult Female Hydrocarbons. Total hydrocarbons from 1- to 5-day-old frass from adult and large nymphal cockroaches and from adult females of unknown age were collected as described previously (Brill and Bertsch, 1985). Frass pellets (200 mg) were immersed in hexane for 24 hr, ground with a glass rod, and rinsed with additional hexane. Thirty adult females were immersed in hexane to remove cuticular lipids. The lipids were reduced in volume by rotoevaporation to 5 ml, and total hydrocarbons were eluted from a silica gel column (1 × 25 cm) with 80 ml hexane.

GC Analysis of Ootheca-, Frass-, and Female-Derived Hydrocarbons. Total hydrocarbons from oothecae, frass, and adult females were analyzed by GC, as were the oothecal hydrocarbon fractions from $AgNO_3$-impregnated silica gel. Additionally, the quantity of 6,9-heptacosadiene was determined for oothecae and frass ($N = 4$ independent total hydrocarbon fractions measured for each) by coinjection of 0.1 μg *n*-hexacosane standard and 0.1 OE of total hydrocarbon or with 0.1 mg equivalent of total hydrocarbon from frass.

Hydrocarbon samples (in 1 or 2 μl hexane) were analyzed with a Tracor gas chromatograph (model 540) equipped with a cool on-column injector, a flame ionization detector (340°C), and hydrogen carrier gas. The capillary column was nonpolar fused silica (DB-1; 30 m × 0.32 mm ID, 0.25-μm film thickness; J & W Scientific, Folsom, California), and the temperature program was 60°C/2 min, 20°C/min to 240°C, held for 0 min, 5°C/min to 320°C, and held for 30 min. Data were recorded with Turbochrom 3 (PE Nelson, Cupertino, California) software with a PE Nelson 900 Series Interface.

Hydrocarbons in this study were identified by chromatogram comparison with published identification of *P. americana* cuticular hydrocarbons (Baker et al., 1963; Beatty and Gilby, 1969; Jackson, 1972), and retention time comparisons with *n*-alkane standards. Baker et al. (1963) and Jackson (1972) identified the alkanes (*n*-pentacosane, 3-methylpentacosane, *n*-heptacosane, and *n*-nonacosane) by their lack of complexing on $AgNO_3$ TLC, response to hydrogenation, inclusion (or not) in a 5 Å molecular sieve, response to bromination, and comparison of GC data from known *n*-alkane standards. 3-Methylpentacosane was further identified by comparison of its mass spectrum with spectra from other cockroach-derived 3-methyl hydrocarbons (3-methyltricosane and 3 meth-

ylheptacosane). Baker et al. (1963), Beatty and Gilby (1969), and Jackson (1972) each identified 6,9-heptacosadiene by its complexing to $AgNO_3$-impregnated silica gel, response to hydrogenation, NMR, IR, UV, and mass spectroscopy. Location of the double bonds was determined by production of both caproic and stearic acids following oxidative cleavage by ozonolysis and potassium-permanganate.

Collection of Volatile 6,9-Heptacosadiene from Oothecae. Compressed air (7.2 liters/hr) was passed through charcoal gravel (#202292, GC hydrocarbon trap grade; Chromatec, Lake Worth, Florida) packed between two, 2-cm-long plugs of highly activated charcoal (Lewcott, Millbury, Massachusetts) in a glass tube (0.6 cm ID). The cleaned air then passed over 175, 1- to 7-day-old oothecae packed into a glass tube (0.6 cm ID). Volatile 6,9-heptacosadiene was collected on 20–30 mg Super Q adsorbent (Alltech, State College, Pennsylvania) held between glass wool plugs and packed into the constriction of a disposable Pasteur pipet attached to the glass tube housing the oothecae. Before volatile collection, the adsorbent was cleaned by elution with 4–5 ml of hexane and dried under a stream of nitrogen. Volatile collections were conducted at 28–30°C. After volatile collection was complete, the adsorbent was eluted with approximately 0.5 ml hexane, concentrated to 0.1 ml, and analyzed by GC-MS. Mass spectral data from volatile 6,9-heptacosadiene was generated on a Perkin-Elmer gas chromatograph (model 8420) interfaced with a Finnigan ion trap. Data were collected and analyzed by INCOS data collection software driven by an IBM-compatible 80286 computer. The capillary column was nonpolar fused silica (DB-1; 30 m × 0.25 mm ID; J & W Scientific). Samples were injected in splitless mode followed, after 30 sec, by a split-purge flow of helium. The helium carrier gas linear flow rate was 25 cm/sec, and the temperature program was 60°C/2 min, followed by 5°C/min to 320°C, and held for 10 min.

RESULTS

Ootheca and Frass Bioassays. A. hagenowii was attracted to volatiles from *P. americana* oothecae and frass. A significantly greater percentage of parasitoids responded to the ootheca at the highest airflow rate than at any of the lower airflow rates (Figure 1). Additionally, at the highest airflow rate parasitoids did not respond when exposed to only air from both lateral arms of the olfactometer; of 100 parasitoids assayed, only one was trapped. *P. americana* frass was also attractive to *A. hagenowii*; 50, 100, and 200 mg of 1- to 5-day-old frass were 97, 124.4, and 96.4% as attractive as an ootheca.

Bioassay of Oothecal Lipids and Hydrocarbons. A. hagenowii was attracted primarily to the hexane fraction (i.e., hydrocarbons) and moderately to the 1%

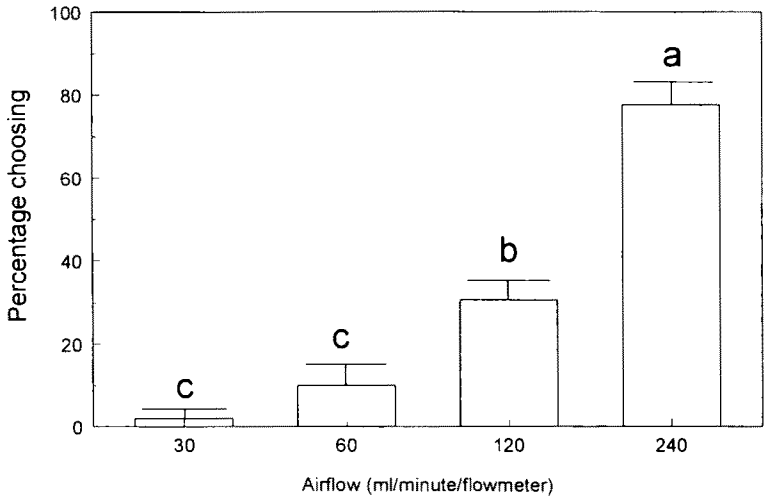

FIG. 1. Ten minute Y-tube bioassay response (mean ± SE, percentage choosing ootheca) of 1-day-old unfed *A. hagenowii* to a < 10-day-old *P. americana* ootheca at each of four airflow rates. $N = 6, 9, 5$, and 5 replicates for the 30, 60, 120, and 240 ml/min/flowmeter airflow rates, respectively. A replicate is the response of 20 insects. Bars topped by different letters are significantly different ($F = 50.4$, $df = 3,45$, $P < 0.0001$) [Tukey's Studentized range test (SAS Institute, 1988)].

ether fraction; fractions eluted with more polar solvent blends were not very attractive (Figure 2A). The hexane and 1% ether fractions were, on average, 77.6 and 19.9%, respectively, as attractive as an ootheca. Bioassay of the hydrocarbon fractions from $AgNO_3$-impregnated silica gel clearly demonstrated that the attractive compound was in the 10% ether fraction (Figure 2B); this fraction was 62.6% as attractive as an ootheca.

GC Analysis of Ootheca-, Frass-, and Female-Derived Hydrocarbons. GC analyses of total hydrocarbon from oothecae, frass, and adult females were qualitatively (Figure 3) and quantitatively (Table 1) very similar; the five dominant peaks constituted 87–96% of the hydrocarbons from each source. The dominant hydrocarbon from each source was 6,9-heptacosadiene (peak 3 in Figure 3). Baker et al. (1963), Beatty and Gilby (1969), and Jackson (1972) had previously found 6,9-heptacosadiene as the major cuticular hydrocarbon on American cockroaches.

GC analysis indicated excellent separation of saturated and unsaturated oothecal hydrocarbons by elution from $AgNO_3$-impregnated silica gel with hex-

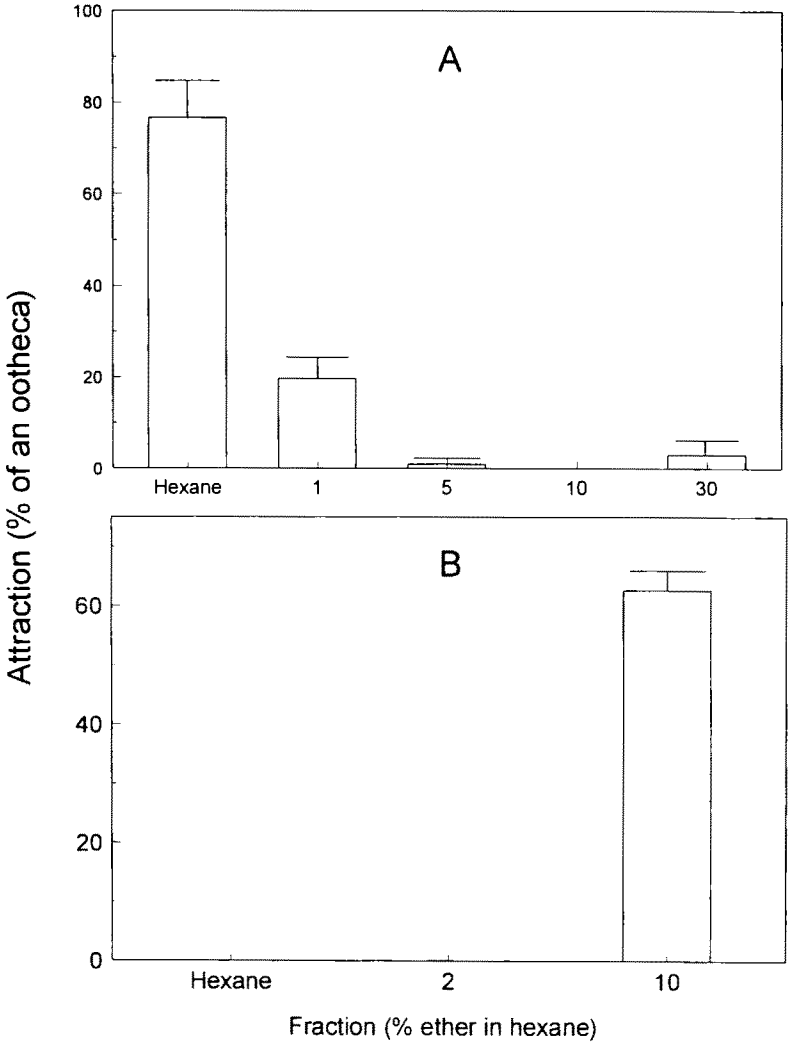

FIG. 2. Attraction (mean percentage of an ootheca ± SE) of 1 OE of (A) cuticular lipids chromatographed on 70–230 mesh silica gel, and (B) cuticular hydrocarbons separated on 10% $AgNO_3$-impregnated silica gel. Attraction is the number of parasitoids responding to a chromatographic fraction divided by the number responding to an ootheca on the same day (i.e., a percentage of the activity of an ootheca). (A) N = 7, 7, 6, 6, and 5 replicates for the hexane, 1, 5, 10, and 30% ether in hexane fractions. (B) N = 6, 6, and 9 replicates for the hexane, 2 and 10% ether in hexane fractions. A replicate is the response of 20 insects.

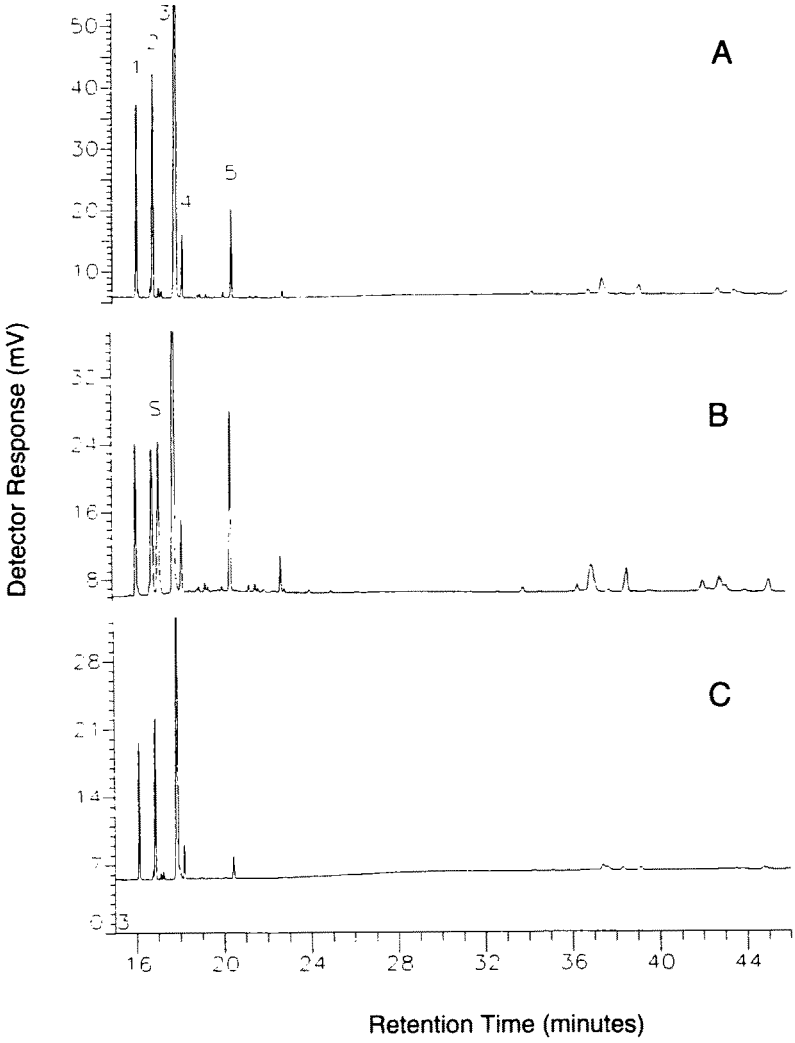

FIG. 3. Gas chromatographic analysis of total hydrocarbons from (A) 1- to 7-day-old *P. americana* oothecae, (B) 1- to 5-day-old frass, and (C) adult females. Hydrocarbon identification after Baker et al. (1963). Peak 1: *n*-pentacosane; 2: 3-methylpentacosane; 3: 6,9-heptacosadiene; 4: *n*-heptacosane; 5: *n*-nonacosane. See Table 1 for corresponding peak area percentages. Peak 3 is off scale to highlight smaller peaks. S is 0.1 μg *n*-hexacosane internal standard. Column: DB-1 (30 m × 0.32 mm ID, 0.25-μm thickness). Temperature program: 60°C/2 min, 20°C/min to 240°C, held for 0 min, 5°C/min to 320°C, and held for 30 min.

TABLE 1. PEAK AREA PERCENTAGE OF HYDROCARBONS FROM *P. americana* OOTHECAE, FRASS, AND ADULT FEMALE

Hydrocarbon source	*N*	% Peak area (mean ± SE)[a]					% of total
		1	2	3	4	5	
Ootheca	5	12.4 ± 0.83	14.7 ± 0.61	60.0 ± 1.04	3.4 ± 0.16	5.5 ± 0.23	96.0 ± 1.71
Frass	4	11.5 ± 1.30	12.9 ± 0.58	45.9 ± 1.10	4.8 ± 0.45	11.9 ± 2.33	87.0 ± 3.45
Female	4	12.9 ± 0.23	15.2 ± 0.18	62.7 ± 0.91	2.9 ± 0.14	3.0 ± 0.06	96.7 ± 0.59

[a] 1 = *n*-pentacosane, 2 = 3-methylpentacosane, 3 = 6,9-heptacosadiene, 4 = *n*-heptacosane, 5 = *n*-nonacosane. Identifications after Baker et al. (1963), Beatty and Gilby (1969), and Jackson (1972).

ane and 2% and 10% ether in hexane (Figure 4). Collectively, Figures 2 and 4 provide strong evidence that the hydrocarbon 6,9-heptacosadiene is the compound responsible for the attraction of *A. hagenowii* to *P. americana* oothecae. This compound was the only one detected in fraction 3 (Figure 4D). There was also a trace amount (2% of total) of diene present in the 2% ether fraction (Figure 4C). The hexane fraction contained only saturated hydrocarbons (Figure 4B).

On a per milligram fresh weight basis, frass contained approximately 37.1 times the amount of 6,9-heptacosadiene as oothecae (surface only). An ootheca extract contained approximately 2.09 ± 0.30 μg 6,9-heptacosadiene, or 0.024 ± 0.003 μg/mg fresh weight (based on an oothecal weight of 87.9 ± 1.29 mg, $N = 9$) and frass contained 0.881 ± 0.10 μg of the alkadiene per milligram fresh weight.

Volatiles. 6,9-Heptacosadiene that volatilized from oothecae was trapped on Super Q (peak 3, Figure 5B). Confirmation of its identity as 6,9-heptacosadiene was determined by several methods. First, via coinjection with alkane standards (*n*-hexacosane and *n*-heptacosane), its Kovats retention index (KI) on a DB-1 column was 2666 (two independent volatile collections); the KI of 6,9-heptacosadiene from *P. americana* oothecae (i.e., Figure 4D) was 2673. In addition, coinjection of oothecal-derived 6,9-heptacosadiene and the volatiles collected from oothecae resulted in elution of a single peak at the retention time of the alkadiene. Finally, the mass spectrum from 6,9-heptacosadiene trapped on Super Q adsorbent was very similar to the spectrum of 6,9-heptacosadiene derived and purified by solvent washing of oothecae; a prominent molecular ion (M^+) of 376 was present in each sample at approximately equal abundance.

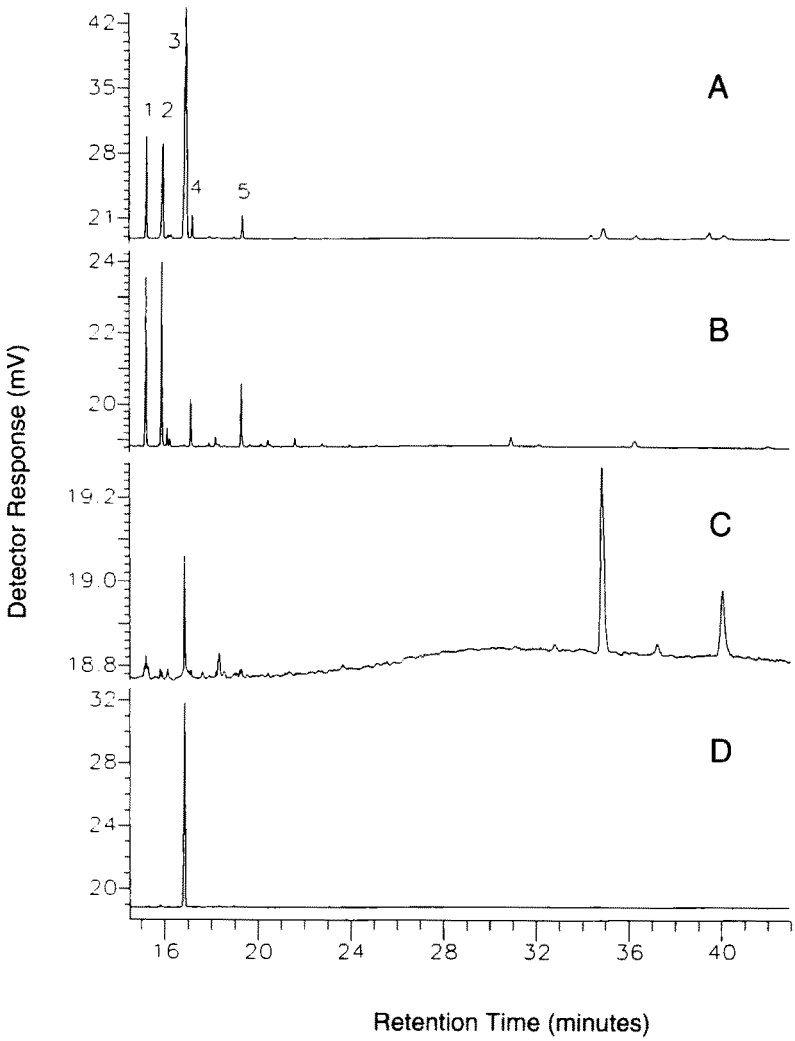

FIG. 4. Gas chromatographic analysis of (A) total hydrocarbons from 1- to 7-day-old *P. americana* oothecae, and oothecal hydrocarbons, which were separated on 10% $AgNO_3$-impregnated silica gel by elution with (B) hexane, (C) 2% ether in hexane, and (D) 10% ether in hexane, with 0.2 OE injected for the hexane, 2%, and 10% ether fractions. Hydrocarbon identification after Baker et al. (1963). Peak 1: *n*-pentacosane; 2: 3-methylpentacosane; 3: 6,9-heptacosadiene; 4: *n*-heptacosane; 5: *n*-nonacosane. The column and conditions are the same as those in Figure 3.

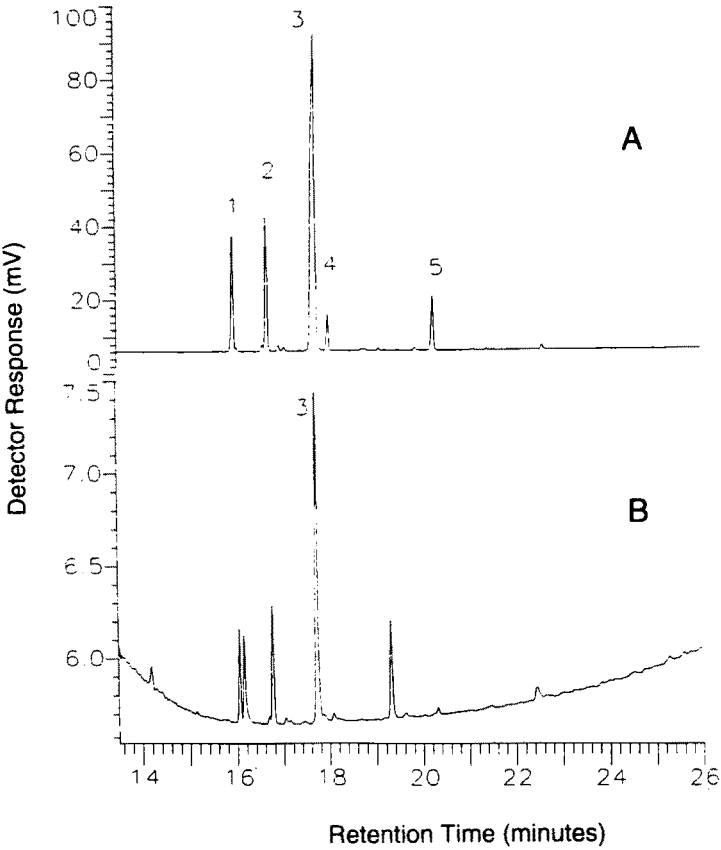

FIG. 5. Gas chromatographic analysis of (A) hydrocarbons from 1- to 7-day-old *P. americana* oothecae, and (B) partial volatiles from < 10-day-old oothecae. Hydrocarbon identification after Baker et al. (1963). Peak 1: *n*-pentacosane; 2: 3-methylpentacosane; 3: 6,9-heptacosadiene; 4: *n*-heptacosane; 5: *n*-nonacosane. The column and conditions are the same as those in Figure 3.

DISCUSSION

The hydrocarbon 6,9-heptacosadiene is used as a short-range kairomone by *A. hagenowii* for locating *P. americana* oothecae. 6,9-Heptacosadiene was identified as a cuticular component in *P. americana* adults (Baker et al., 1963) and is the most abundant lipid constituent of cuticle. Hydrocarbons account for the majority (75–95%) of cuticular lipids in *P. americana* (Gilby and Cox,

1963; Jackson, 1972), and 6,9-heptacosadiene makes up approximately 65–70% of its total hydrocarbon composition. In the present study, total hydrocarbon composition from oothecae, frass, and adult females were qualitatively and quantitatively similar, and paralleled earlier analyses of the hydrocarbon composition of adult *P. americana* (Baker et al., 1963; Beatty and Gilby, 1969; Jackson, 1972).

The current study is the first evidence of biological activity of 6,9-heptacosadiene. Alkadienes with behavioral activity have been found in other insects. For example, 7,11-heptacosadiene, found in the cuticular wax of female *Drosophila melanogaster* (Antony and Jallon, 1982; Antony et al., 1985), has activity in mating bioassays, and 6,9-nonadecadiene is a component of the sex pheromone of several geometrid moth species (Szocs et al., 1984; McDonough et al., 1986). Bartelt et al. (1982) identified a series of 9,19-alkadienes (C_{28}–C_{39}) as the sex pheromone blend of a sawfly, *Pikonema alaskensis*.

The identification of a kairomone in the frass and cuticle of a host was first documented by Jones et al. (1971), who identified 13-methylhentriacontane in the frass, larvae, saliva, and hemolymph of *Heliothis zea* as a host-seeking cue for its parasitoid *Microplitis croceipes*. *P. americana*-infested harborages often contain many kairomone-laden materials such as frass, oothecae, exuviae, and dead cockroaches. The kairomone in these materials may help to maintain a high density of parasitoids in the cockroach breeding area. Lewis et al. (1972) significantly enhanced the parasitism of Lepidoptera eggs by *Trichogramma evanescens* in Petri dishes, greenhouses, and cotton fields by prerelease application of a hexane-soluble host-location kairomone, later identified as *n*-tricosane, from moth scales. Eggs placed in locations treated with the extract suffered higher parasitism than control eggs (i.e., no kairomone). In a later study, Lewis et al. (1975) discovered that broadcast application of *n*-tricosane resulted in increased parasitism of sentinel and wild Lepidoptera eggs by wild and released *Trichogramma* spp. Unlike the inundative release of sex pheromone for mating disruption, large-scale application of a host-seeking stimulant did not confuse the parasitoids; it stimulated host-searching, resulting in increased parasitism.

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RESPONSE OF XYLEM-FEEDING LEAFHOPPER TO HOST PLANT SPECIES AND PLANT QUALITY

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Abstract—*Carneocephala floridana*, an oligophagous leafhopper that inhabits the salt marshes along the coasts of Florida, utilizes *Borrichia frutescens* and *Salicornia virginica* (both herbs) as primary summer hosts, but uses two grasses, *Distichlis spicata* and *Spartina alterniflora*, during the winter. We tested whether the seasonal patterns of abundance and apparent host-switching by *Carneocephala* are related to plant quality. In laboratory experiments, nymphs of *Carneocephala* reared on nonfertilized control plants of the two herbs produced adults that were similar in size to field-collected insects. Only *Carneocephala* raised at the lowest densities on *Spartina* and *Distichlis* from the highest fertilizer treatments produced adults similar in body mass to those reared on nonfertilized *Borrichia* and *Salicornia*. For *Distichlis*, superior quality (high foliar nitrogen) plants were able to mitigate the negative effect of nymphal crowding on adult body mass. However, laboratory fertilization regimes produced an extremely high foliar nitrogen content in the two herbs and the organic acid concentration in the xylem fluid of *Borrichia*, the only host species suitable for xylem fluid extraction, increased 2.5- to 3-fold. Total amino acid concentration in the xylem fluid of fertilized *Borrichia* decreased compared to nonfertilized plants. *Carneocephala* demonstrated reduced feeding efficiencies on high nitrogen *Borrichia*. Our results suggest that *Carneocephala* prefers, and performs better on, plants with high nitrogen content up to a threshold, beyond which high nitrogen levels result in reduced leafhopper feeding rates and assimilation efficiencies.

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Key Words—*Carneocephala floridana*, leafhopper, nitrogen content, amino acids, organic acids, insect nutrition, preference, performance.

INTRODUCTION

Laboratory studies have revealed that peak insect performance occurs at nitrogen levels that are well above those found in most host-plant tissues (McNeill and Southwood, 1978; Mattson, 1980). Moreover, the form of host plant nitrogen is crucial to insect performance (Strong et al., 1984; Brodbeck and Strong, 1987). Plant secondary chemicals may have a significant negative effect on host-plant utilization and insect performance, even when total nitrogen or soluble protein are found in high concentrations within the plant's tissues (Feeny, 1970).

Secondary chemicals probably have the greatest impact on folivores because they are housed within the vacuoles or other organelles of the leaf cells (McKey, 1979). As a result, secondary chemicals may mask the direct nutritional values of host tissues on insect performance. Unlike leaf-chewers, xylem-feeders provide an excellent opportunity to study the nutritional effects of amino acid levels on insect preference and performance, while largely eliminating interactions with plant secondary compounds (Brodbeck et al., 1990). Xylem sap is primarily composed of water (typically around 98%) and the majority of organic solutes are amino and organic acids (Mattson, 1980; Andersen and Brodbeck, 1989; Andersen et al., 1989). Although relatively few secondary chemicals are found in the xylem fluid, the concentration of nitrogen-containing compounds is often two to three orders of magnitude lower than those found in the leaf tissues, which are in turn below the levels required for maximum insect performance (Mattson, 1980; Brodbeck et al., 1993).

How then do xylem feeders survive on such a dilute diet? Leafhoppers have developed several adaptations that have enabled them to specialize on xylem fluid. First, they have a highly modified oral cavity (the cibarial pump), which allows them to pull against the negative pressure of the xylem vessels (Backus, 1985). Moreover, they are very efficient feeders. For example, Brodbeck et al. (1993) found that three species of xylophagous leafhoppers had assimilation efficiencies of 99% and 94% for carbon and nitrogen, respectively.

Carneocephala floridana, an oligophagous xylem-feeding leafhopper, is a common inhabitant of salt marshes along Florida's coastal regions (Ball, 1927; Nottingham, 1932; Rossi and Strong, 1990). *Carneocephala* shows distinct seasonal patterns on its four primary hosts, which include two herbs, *Borrichia frutescens* and *Salicornia virginica*, and two grasses, *Spartina alterniflora* and *Distichlis spicata*. In northwest Florida, *Carneocephala* utilizes the herbs as its primary hosts during most of the year, but in the winter when the herbs experience an extensive dieback, the leafhopper switches to the grasses (Rossi, 1991;

Rossi et al., 1994). Although seasonal differences of interspecific host-plant nitrogen content were not good predictors of host choice by *Carneocephala*, temporal abundance of leafhoppers on each plant species was more closely correlated with intraspecific nitrogen content (Rossi, 1991). In both laboratory and field-plot studies, we have found that *Carneocephala* shows a preference for, and performs better on, superior quality (i.e., higher nitrogen) culms of *Spartina* than on low-nitrogen culms (Rossi, 1991; Rossi, and Strong, 1991).

In the present study, we investigated the effect of fertilization on the abundance of *Carneocephala* in field-plot monocultures of *Spartina* and *Borrichia*. In the laboratory, we examined the influence of host-plant nitrogen level and insect crowding on nymphal performance. Lastly, we extracted the xylem fluid from one suitable species, *B. frutescens*, grown under different fertilization regimes, which allowed us to relate specific dietary components to insect performance. Understanding these relationships may help to explain the selective advantages of seasonal shifts in host-plant specificity exhibited by *Carneocephala*.

METHODS AND MATERIALS

Interspecific Host Choice. Simultaneous laboratory choice experiments were performed during the summer and winter to assess interspecific host-plant preference of *Carneocephala* according to the methods of Rossi and Strong (1991). Several hundred small shoots or culms of the four host-plant species were collected and grown in a greenhouse for approximately three months prior to the choice experiments. Plants of similar wet mass were randomly assigned to one of four positions within Plexiglas choice arenas. One day prior to the choice experiments, several hundred female *Carneocephala* were collected from a patch of mixed vegetation, which consisted of the four major hosts, from Goose Creek, Wakulla County, Florida. Ten female leafhoppers were released into each arena, and the number of insects present on each plant was recorded after 24 and 48 hr. After 48 hr, the insects were removed from the arenas, and the total number of eggs laid as well as per capita rate of egg production was calculated for each plant species. The experiment was replicated 10 times. The proportions of leafhoppers on each plant, after 48 hr, were arcsine-square-root transformed and then analyzed using a one-way ANOVA (Sokal and Rohlf, 1969). Differences among host species for total number of eggs and per capita egg production were assessed by a one-way ANOVA on log-transformed egg counts. When appropriate, comparisons among treatments were subjected to Tukey's HSD post-hoc test for separation of means (Petersen, 1985).

Insect Performance. Carneocephala were reared from first instar to adult on differentially fertilized *Borrichia*, *Distichlis*, and *Salicornia* to determine the

relationship among insect survivorship, body mass, and host nitrogen. The fertilizer solutions were identical to those in Rossi and Strong (1991) except nitrogen concentrations, in the form of NH_4NO_3, used in the current study were 0 (control), 60 (low), and 200 (high) mg/liter.

Leafhoppers were maintained in acetate tubes (5.5 cm OD), which completely enclosed the plant and were sealed at the top with organdy cloth. This method allowed the nymphs to choose their own feeding site. Like many species of homopterans, *Carneocephala* nymphs typically feed in small groups. Although the reasons for this behavior in *C. floridana* are unknown, gregarious feeding, often seen in many species of aphids (Mittler, 1957; Dorschner et al., 1987), may produce a sink for plant resources, benefiting the group members, or it may be that there are a limited number of preferred feeding sites on the plant. To assess effects of crowding on leafhopper performance, nymphs were placed in groups of either two or five nymphs per plant. This design enabled us to measure the effects of host-plant nitrogen content (quality) and leafhopper crowding on development time and dry mass at eclosion. The proportion of leafhoppers surviving was arcsine-square-root transformed and the data were analyzed using a two-way ANOVA with plant nitrogen content and leafhopper density as main effects.

Fertilized Field Plots and Laboratory Experiments with Borrichia. Plots of Borrichia were fertilized with NH_4NO_3 at two sites: Kornegay and Southsides at Oyster Bay, Florida, according to the methods described in Rossi et al. (1992). This allowed us to investigate the importance of plant nitrogen on *Carneocephala* host preference under field conditions and compare the results to those from *Spartina* (from Rossi, 1991; Rossi and Strong, 1991). At each site, four 2-m^2 plots were marked at the corners using polyvinylchloride (PVC) stakes. Plots were paired and separated by approximately 1 m. One plot in each pair was randomly assigned to the control treatment and received no fertilizer; the other plot received approximately 530 g/m^2 of fertilizer, broadcast on January 13 and March 8, 1990. Nitrogen as a percentage of total dry mass was determined in duplicate with an elemental analyzer (model 1600, Carlo Erba, HBI, Saddle Brook, New Jersey) using 10 leaves collected from the field plots on May 13, 1991. Leaf samples were placed in plastic bags and kept on Dry Ice until they were returned to the laboratory. Samples were stored at $-60°C$ until analysis. Prior to nitrogen analysis, the samples were freeze-dried for 72 hr and then ground in a Wiley mill (see Rossi and Strong, 1991; Rossi et al., 1992, for full details).

Trends in *Carneocephala* abundance were monitored using standard 3 × 5-in. (7.6 × 12.7-cm) index cards. The cards were held in place on PVC stakes with clips and placed just above the tops of the plants. The cards were painted with a bright Day-Glo yellow paint and wrapped in an acetate sheet that was

coated with Tangle-Trap (The Tanglefoot Co., Grand Rapids, Michigan), and are referred to as yellow sticky traps (YSTs). Traps were changed approximately every two to three weeks for one year beginning in February 1990. Because fertilized plants eventually become larger than control plants, the number of leafhoppers captured on the YSTs in the fertilized plots would be expected to be greater than those captured on the traps in the control plots because fertilized plots contain more biomass and, hence, should support more insects. Therefore, we converted the number of *Carneocephala* captured on the YSTs to a standardized "hopper load." The hopper load was calculated by dividing the number of hoppers on the YST for a given plot by the average leaf area, determined with a leaf area meter (model CI-201, CID, Inc., Moscow, Idaho 83843), of 20 haphazardly-chosen leaves collected from each plot on October 30, 1990. Because leaf area was determined late in the growing season and well after fertilization in the treatment plots when the treatment leaves were much larger than those from control plots (see Rossi et al., 1992 for details), the method was conservative.

To supplement the performance studies and field experiments with *Borrichia*, we examined the effects of intraspecific host-plant nitrogen in the laboratory using a subset of differentially-fertilized *Borrichia*. *Borrichia* was the only host used in this experiment because it is possible to collect and analyze its xylem fluid, which allowed for measurement of nutrient intake by feeding *Carneocephala* (described in the next section). A single plant of *Borrichia* from each of the three previously described fertilizer treatment groups was randomly assigned a position within the choice arenas (the remaining position was occupied by a sand-filled pot). Ten adult females were released into each arena, analogous to the methods used for the interspecific experiments, and the number of hoppers on each plant was recorded after 24 and 48 hr. The experiment was replicated six times. Because the plants were destructively sampled after 48 hr to search for eggs, the proportion of leafhoppers on each plant was divided by the mass of the plant to produce a hopper load, which provided a more precise measure of leafhopper preference than the hopper load calculated from the field plots.

Chemical Profile of Insect Exudate and Borrichia *Xylem Fluid.* Individual adult female leafhoppers were maintained in small clip cages 2 cm OD × 4 cm long and sealed at both ends with plastic lids. The cages were placed over the stems of *Borrichia*. A 1.5-ml Eppendorf tube was placed through a hole in the bottom lid to collect the exudate (excreta) produced by the insects. Leafhopper feeding rates were determined on plants from each treatment group by measuring the amount of excreta collected in clip cages in a 24-hr period. Excreta was collected using a 25-μl pipet. Because exudate was very dilute, it was necessary to pool the excreta produced from insects feeding on several plants within a

group to provide sufficient quantities for chemical analyses. The exudate was stored at −60°C until analysis.

Xylem fluid was extracted at night, when xylem tensions are typically highest (least negative) (Salisbury and Ross, 1985), from 10- to 20-cm-long shoots of *Borrichia* using a pressure chamber apparatus (Scholander et al., 1965; Andersen et al., 1989). The xylem tissue was exposed by peeling back tissue exterior to the xylem ring. Xylem fluid was extracted by variable pressure ranging from 0.5 to 1.5 MPa for up to 3 min. The fluid was collected with a 5-μl pipet and stored at −60°C until analysis. As with the exudate, it was necessary to pool xylem fluid from several plants within a treatment group to provide sufficient quantities for chemical analyses. Both excreta and xylem fluid were collected during the two weeks of the performance experiments, and thus a replicate was the pooled exudate and xylem fluid for a week ($N = 2$).

Samples of xylem fluid and insect excreta were centrifuged at 12,000*g* for 20 min. The supernatant was collected, filtered through a 0.22-μm mesh, and then split into equal aliquots for amino acid and sugar/organic acid analyses. Samples used for amino acid analysis were hydrolyzed with 6 M HCl at 110°C for 24 hr under a nitrogen atmosphere and then freeze-dried. Samples were derivatized prior to amino acid analysis by adding 100 μl of 2:2:1 ethanol–triethanolamine (TEA)–H_2O, drying under a vacuum, and than adding 20 μl of 7:1:1:1 ethanol–TEA–H_2O–phenylisothiocyanate (PITC) to each sample. The derivatizing mixture was allowed to react for 20 min under a nitrogen atmosphere at 22°C. Samples were dried and reconstituted in a phosphate buffer containing 5 mM sodium phosphate with 6% acetonitrile. Samples and external amino acid standards were quantified with a Waters Associate HPLC gradient system equipped with a Pico Tag column (Heinrickson and Meredith, 1984; Andersen et al., 1989).

Organic acids were analyzed by cation exchange chromatography on a Waters 600 HPLC system (modified from Turkelson and Richards, 1978). Samples in 0.01 N H_2SO_4 buffer were run isocratically through an Ion-300 polymeric cation exchange column at 37°C. Organic acids were monitored at a wavelength of 214 nm. Sugars were quantified by a Waters refractive index detector (model R401).

RESULTS

Interspecific Host Choice: Summer Trials. During the summer, *Carneocephala* showed a strong group preference for the two herbs and a moderate preference for *Spartina* ($F = 6.57$; $df = 3,36$; $P = 0.001$) (Table 1). Although total eggs laid per plant differed significantly among the host plants ($F = 12.01$; $df = 2,27$; $P < 0.001$), per capita egg production was not significantly affected

TABLE 1. PROPORTION OF FEMALES, TOTAL NUMBER OF EGGS, AND PER CAPITA NUMBER OF EGGS/FEMALE LAID BY *Carneocephala floridana* ON ITS MAJOR HOST PLANTS[a]

Host species	Proportion	Total eggs	Per capita no. eggs
Summer			
Borrichia frutescens	0.30 ± 0.04ab	12.2 ± 1.1a	3.0 ± 0.5
Distichlis spicata	0.14 ± 0.04c	0.6 ± 0.2[b]	0.1 ± 0.1[b]
Salicornia virginica	0.36 ± 0.03a	12.5 ± 1.3a	1.7 ± 0.2
Spartina alterniflora	0.20 ± 0.03bc	4.8 ± 1.3b	2.9 ± 0.8
Winter			
Borrichia frutescens	0.39 ± 0.05a	18.1 ± 2.0a	3.4 ± 0.3a
Distichlis spicata	0.34 ± 0.05ac	6.1 ± 1.6b	1.3 ± 0.3c
Salicornia virginica	0.10 ± 0.03b	18.0 ± 1.9a	9.2 ± 0.9b
Spatina alterniflora	0.17 ± 0.04bc	7.3 ± 1.8b	2.8 ± 0.7c

[a] Values are mean ± SE. Means followed by different letters within each column are significantly different at $\alpha = 0.05$.
[b] Eggs laid on this species not used in calculation of ANOVA.

by plant species ($F = 1.79$; $df = 2,27$; $P = 0.19$). Feeding and oviposition on *Distichlis* was low. In fact, so few eggs were laid on *Distichlis* that it had to be dropped from egg analyses. While the total number of eggs laid on the two herbs averaged over 12 eggs/plant, the total laid on *Spartina* averaged only 5 eggs/plant.

Winter Trials. Host-plant species had a significant effect on leafhopper preference in the winter choice experiment ($F = 7.23$; $df = 3,36$; $P = 0.001$). While *Carneocephala* continued to show a strong preference for *Borrichia*, it also settled on *Distichlis* in large numbers (Table 1). Only 10% of the leafhoppers settled on *Salicornia* compared to 36% in the summer. Females continued to lay the majority of their eggs on the herbs ($F = 12.83$; $df = 3,36$; $P < 0.001$). Moreover, per capita egg production was highest on the two herbs ($F = 25.05$; $df = 3,36$; $P < 0.001$), especially on *Salicornia*, which averaged 9.2 eggs/female/day.

Insect Performance. In the laboratory, survivorship of *Carneocephala* from first instar to adult was low on the two fertilized treatments of both herbs (Figures 1 and 2). As a result, these two species (and the species main effect) had to be dropped from the calculation of the ANOVA. Therefore, only data collected using *Distichlis* (Figure 3) were used to analyze the effects of host-plant nitrogen and juvenile crowding on adult body mass of *Carneocephala*.

Nitrogen content had a significant effect on dry adult body mass at eclosion ($F = 9.784$; $df = 2$; $P < 0.001$), but the sexes did not respond equally ($F =$

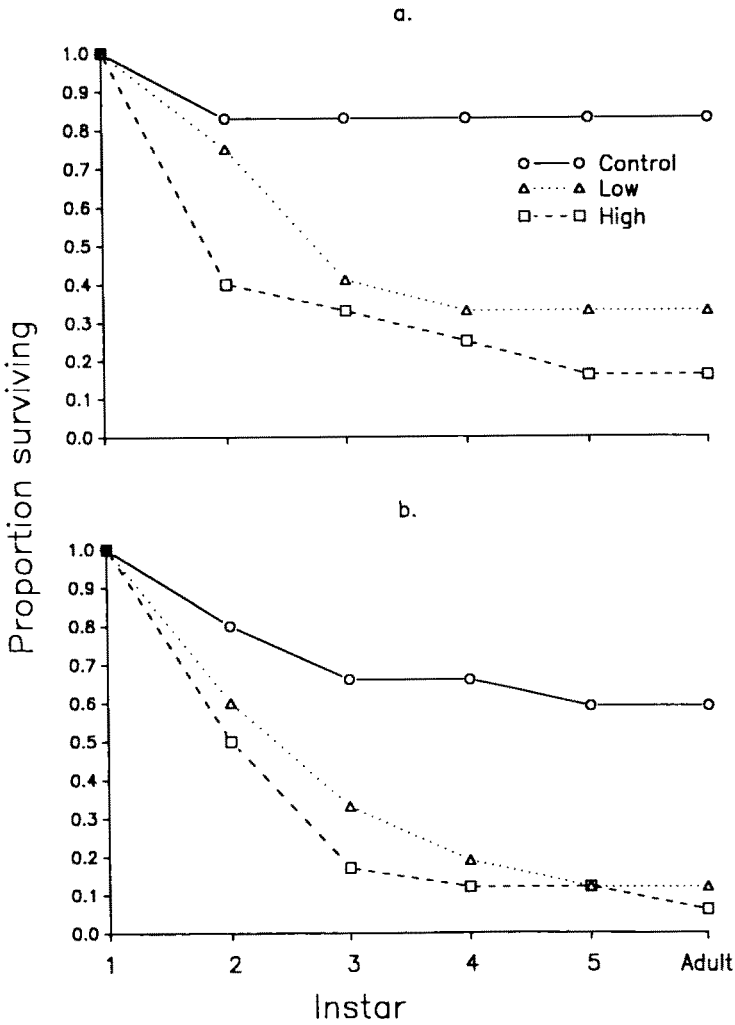

FIG. 1. The proportion of *Carneocephala floridana* surviving from first instar to adult on differentially fertilized *Salicornia virginica* at two densities: (a) 2 nymphs/plant and (b) 5 nymphs/plant.

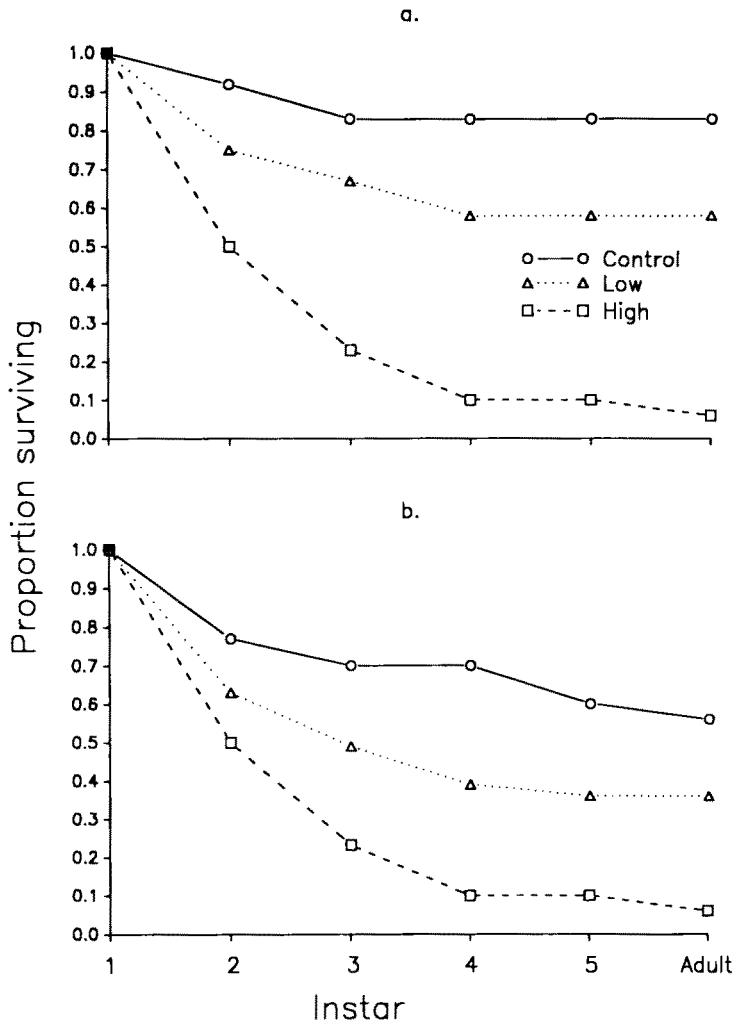

FIG. 2. The proportion of *Carneocephala floridana* surviving from first instar to adult on differentially fertilized *Borrichia frutescens* at two densities: (a) 2 nymphs/plant and (b) 5 nymphs/plant.

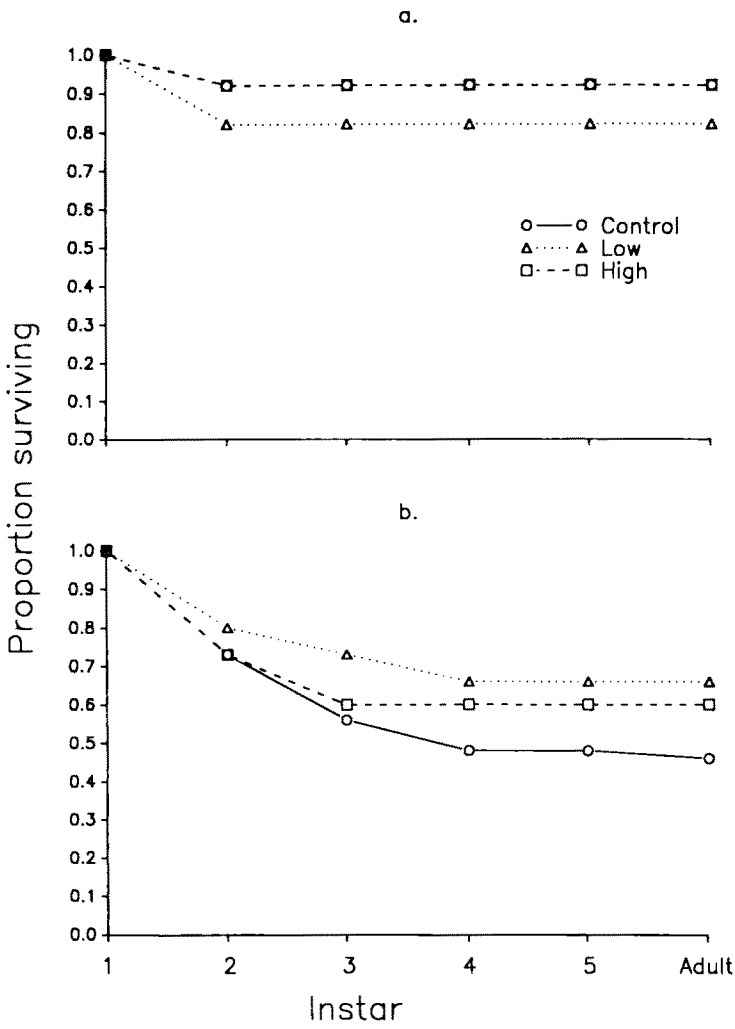

FIG. 3. The proportion of *Carneocephala floridana* surviving from first instar to adult on differentially fertilized *Distichlis spicata* at two densities: (a) 2 nymphs/plant and (b) 5 nymphs/plant.

80.899; $df = 1$; $P < 0.001$). Nymphal density had a highly significant effect on adult body mass ($F = 10.128$; $df = 1$; $P = 0.003$). Leafhoppers reared at 2 nymphs/culm were consistently larger than those reared at 5 nymphs/culm (Figure 4). Although both males and females showed a positive increase in body mass on fertilized plants at low densities, females showed a 77% increase in body mass as plant nitrogen increased from 1.9% to 2.5%, while males only demonstrated an 18% increase. For both males and females, the positive influence of host-plant nitrogen content on body mass was reduced for leafhoppers reared at high density (11 and 21% for males and females, respectively). Development time was not affected by nitrogen level of the host plant or nymphal density.

Fertilized Field Plots and Laboratory Experiments with Borrichia. Fertilization had a significant effect on the percent nitrogen content of *Borrichia* in field plots ($F = 26.52$; $df = 1,2$; $P = 0.04$; see Rossi et al., 1992, for full details). Mean nitrogen content for control and fertilized plots were 1.37 ± 0.010% and 2.95 ± 0.23%, rspectively. Moreover, hopper loads in fertilized plots at both sites were significantly higher than those of controls ($F = 92.243$; $df = 1$; $P = 0.001$). The only other significant effect was that of time ($F = 3.381$; $df = 13$; $P = 0.001$), which reflected seasonal patterns of *Carneocephala*'s abundance (Figure 5).

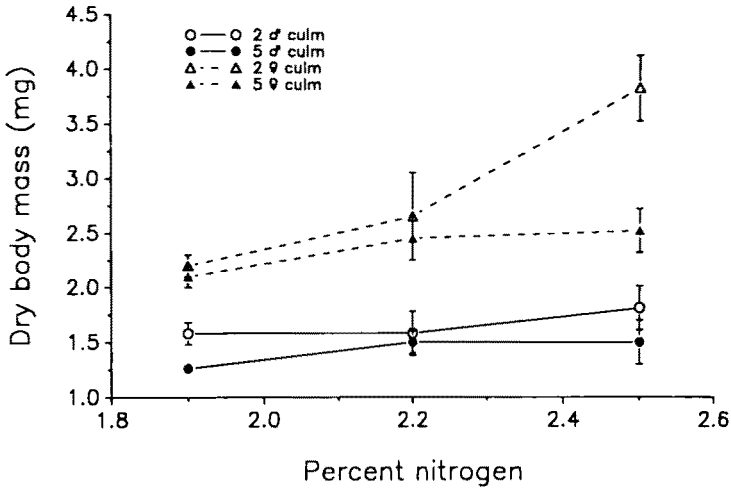

FIG. 4. Mean adult dry body mass of male and female *Carneocephala floridana* reared at two densities on differentially fertilized *Distichlis spicata* having variable nitrogen content. Bars are standard errors.

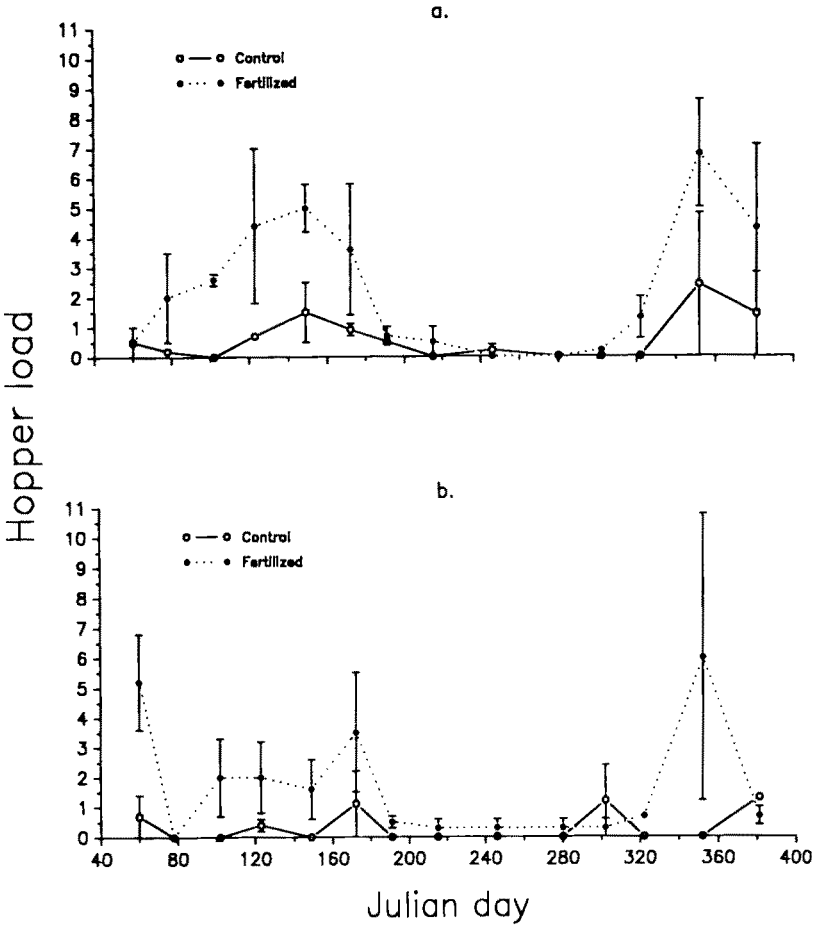

FIG. 5. *Carneocephala floridana* hopper loads (see text) from control and fertilized field plots of *Borrichia frutescens* at two sites: (a) Kornegay and (b) Southsides.

In laboratory trials, fertilization treatment also had an effect on preference of *Carneocephala*. This is evident in the significantly lower hopper loads ($F = 5.64$; $df = 2,14$; $P = 0.02$), and reduced exudate output ($F = 7.13$; $df = 2,42$; $P = 0.002$) on the two fertilized groups of *Borrichia* (Table 2). While the total number of eggs laid was not significantly affected by the application of fertilizer ($F = 2.37$; $df = 2,15$; $P = 0.13$), the per capita rate of egg production was

TABLE 2. EFFECT OF LABORATORY FERTILIZATION REGIMES ON NITROGEN CONTENT OF *Borrichia frutescens* AND PREFERENCE OF *Carneocephala floridana*[a]

Fertilizer treatment	Percent nitrogen	Volume (ml) exudate/24 hr	Hopper load	Total eggs	Per capita eggs
Control	1.9 ± 0.1a	0.20 ± 0.03a	3.3 ± 0.1a	14.5 ± 2.2	4.0 ± 0.7a
Low	4.5 ± 0.2b	0.15 ± 0.04ab	1.7 ± 0.3ab	8.3 ± 2.8	1.8 ± 0.5b
High	5.0 ± 0.1b	0.07 ± 0.01b	0.9 ± 0.3b	7.0 ± 2.8	1.6 ± 0.6b

[a] Values are mean ± SE. Means followed by different letters within the same column are significantly different at $\alpha = 0.05$.

TABLE 3. MEAN AMINO ACID CONCENTRATIONS IN XYLEM FLUID OF DIFFERENTIALLY FERTILIZED *Borrichia frutescens* AND EXUDATE PRODUCED BY FEEDING *Carneocephala floridana*[a]

	Fertilization treatment					
	Control		Low		High	
Amino acid	Xylem	Exudate	Xylem	Exudate	Xylem	Exudate
Asp	172	0.52	5	3.23	ND	3
Glu	134	ND	10	7.65	ND	ND
Ser	71	0.53	ND	0.78	40	ND
Gly	61	ND	ND	3.67	ND	1.08
His	10	ND	21	0.96	11	2.29
Arg	24	ND	30	0.67	26	ND
Thr	22	ND	ND	0.32	ND	ND
Ala	73	ND	ND	2.13	6	0.75
Pro	60	2.40	112	5.32	78	6.27
Tyr	ND	0.78	ND	0.56	ND	2.22
Val	13	0.42	15	1.13	17	0.62
Cys	ND	ND	ND	0.39	ND	0.57
Met	ND	0.71	6	0.40	5	2.60
Ile	10	0.42	20	1.70	12	2.15
Leu	18	ND	ND	1.09	5	0.18
Phe	4	ND	ND	0.31	3	ND
Lys	6	ND	ND	0.85	5	0.49
Total	678	5.78	219	31.06	208	22.22

[a] Results are from two pooled samples. All concentrations are given as micromolar. ND = not detectable in xylem fluid < 4 μM and in exudate < 0.25 μM.

altered ($F = 5.28$; $df = 2,15$; $P = 0.02$). Individually, female *Carneocephala* laid fewer eggs on the two fertilized groups.

Chemical Profile of Insect Exudate and Borrichia *Xylem Fluid.* Amino acid concentration of the xylem fluid varied greatly among plants receiving differing levels of nitrogen fertilizer (Table 3). Abundance of *Carneocephala* was higher on nonfertilized control plants, which had amino acid concentrations approximately three times *higher* than those of the fertilized groups, despite the fact that fertilized plants were 2–2.5 times higher in total nitrogen content (see previous section). Moreover, assimilation efficiency of amino acids (i.e., percent organic compounds extracted during feeding) was >99% on control plants; assimilation efficiency dropped to 85 and 89% on low and high fertilized plants, respectively. The amino acid profile of nonfertilized *Borrichia* consisted primarily of asparagine and glutamine. Conversely, proline was especially high in the amino acid profiles of the two fertilized treatments and asparagine and glutamine were low. Although NH_4^+ was not quantified, a large increase in the peak that corresponds to ammonium was observed for fertilized plants compared to nonfertilized control plants.

In contrast to the amino acid profiles, the organic acid concentrations of the xylem fluid of plants from the two fertilized treatments increased threefold over the control plants (Table 4). Tartaric, citric, and malic acids showed the greatest increases with the addition of fertilizer. Feeding efficiencies of organic acids by *Carneocephala* on plants from the three groups were similar; 47, 49, and 41% for control, low, and high treatments, respectively.

TABLE 4. MEAN ORGANIC ACID CONCENTRATIONS IN XYLEM FLUID OF DIFFERENTIALLY FERTILIZED *Borrichia frutescens* AND EXUDATE PRODUCED BY FEEDING *Carneocephala floridana*[a]

Organic acid	Fertilization treatment					
	Control		Low		High	
	Xylem	Exudate	Xylem	Exudate	Xylem	Exudate
Oxalic	27	13	19	23	26	28
Tartaric	240	222	1231	520	540	444
Citric	622	285	1123	560	2081	992
Malonic	ND	ND	ND	ND	ND	ND
Malic	359	140	664	436	1092	756
Succinic	ND	ND	ND	ND	ND	ND
Total	1248	660	3037	1539	3739	2220

[a]Results are from two pooled samples. All concentrations are given as micromolar. ND = not detectable in xylem fluid < 10 μM and in exudate < 2.5 μM.

TABLE 5. MEAN SUGAR CONCENTRATIONS IN XYLEM FLUID OF DIFFERENTIALLY FERTILIZED *Borrichia frutescens* AND EXUDATE PRODUCED BY FEEDING *Carneocephala floridana*[a]

	Fertilization treatment					
	Control		Low		High	
Sugar	Xylem	Exudate	Xylem	Exudate	Xylem	Exudate
Glucose	129	43	195	36	63	44
Fructose	158	45	220	93	291	42
Sucrose	288	38	126	ND	137	ND
Total	575	126	541	129	591	83

[a]Results are from two pooled samples. All concentrations are given as micromolar. ND = not detectable in xylem fluid < 5 μM and in exudate < 1.25 μM.

Lastly, sugar concentrations, which ranged from 0.5 to 0.6 mM, showed little response to the addition of fertilizer (Table 5). Feeding efficiencies of sugars by *Carneocephala* were 78, 76, and 86% for control, low, and high fertilizer treatments, respectively.

DISCUSSION

While *Borrichia* and *Salicornia* were the preferred hosts for both feeding and oviposition in the summer, this pattern showed dramatic changes during the winter trials. In the winter, *Carneocephala* still showed a strong preference for *Borrichia* as a feeding and ovipositional substrate, but it also exhibited a strong feeding orientation to *Distichlis*, largely at the expense of *Salicornia*. Interestingly, while the feeding preference for *Salicornia* dropped by more than 70% in the winter, this succulent remained a preferred ovipositional host for the leafhopper.

Both *Borrichia* and *Salicornia* experience a substantial dieback during the winter months (Rossi and Strong, 1991; Rossi et al., 1994). The leaves on *Borrichia* generally remain intact until the first hard frost. Dieback for this species appears to be largely temperature mediated since it actively grows throughout the winter in protected sites. In contrast, *Salicornia* gradually loses its green color and turns bright red in the winter, which is the consequence of the breakdown of chlorophyll and the production of anthocyanins on cool days (Salisbury and Ross, 1985). Thus, although *Salicornia* did not die back in the greenhouse during the winter, some chemical changes (apparently controlled by

day length) occurred that may have reduced its palatability to *Carneocephala*. However, the soft succulent stems were still acceptable to the leafhopper during the winter as an ovipositional substrate.

Experiments in which we reared *Carneocephala* nymphs at different densities and on differentially-fertilized *Distichlis* suggest that feeding on high-nitrogen plants moderated the negative effect of juvenile crowding on resultant adult body mass. This pattern of increased adult size was also observed during experiments in which *Carneocephala* were reared on fertilized *Spartina* (Rossi and Strong, 1991). Denno et al. (1986) also found that rearing the planthopper, *Prokelisia marginata*, on high-nitrogen ("superior quality") *Spartina* offset the negative effects of high insect densities on adult body length.

If high-nitrogen plants are generally beneficial to leafhopper growth and suvivorship, why did so few *Carneocephala* survive on fertilized *Borrichia* and *Salicornia*? Moreover, why did *Carneocephala* show less preference for fertilized *Borrichia* as both feeding and ovipositional hosts in laboratory experiments than it did for nonfertilized control plants? These results seem contradictory to field studies in which *Carneocephala* showed significantly higher hopper loads in fertilized plots. This apparent discrepancy can be explained by the fact that *Borrichia* in fertilized field plots had a nitrogen content of only 2.9% (compared to 1.4% for control plants) (Rossi and Strong, 1991), while the low- and high-fertilized groups of *Borrichia* reared in the laboratory produced foliar nitrogen levels of 4.5 and 5.0%, respectively. In addition, the extremely high nitrogen levels in the laboratory plants actually resulted in lower total amino acid concentrations in their xylem fluid. Fertilized lab plants had highly unbalanced amino acid profiles, in which proline became the predominant amino acid constituent (comprising 51 and 38% of the total amino acid concentration for the low- and high-fertilizer plants, respectively, compared to 9% for the nonfertilized plants). Studies using artificial diets have shown that highly unbalanced diets of amino acids may be unusable or even toxic to insects (Reese, 1979; Brodbeck et al., 1990). The high mortality rates and low feeding efficiencies of the leafhoppers reared on fertilized plants in laboratory suggests that one, or both, of these processes may have been operating on *Carneocephala*.

Total organic acid concentrations showed large increases in the two fertilized treatments. Fertilization typically results in increased organic acid concentrations, which serve to counter the increase in positively charged ions (Andersen et al., 1989). This increase in the organic acid titer also may have been responsible for the decrease in the preference of *Carneocephala* for fertilized plants. For example, oxalic acid has been demonstrated to be a feeding deterrent for the brown planthopper (Sōgawa, 1982), and it was the least efficiently assimilated organic compound ingested by three species of xylophagous leafhoppers (Brodbeck et al., 1993).

Increases in host-plant nitrogen, up to a critical level, appear to be correlated with increased preference and performance of *Carneocephala*. However, very high levels of nitrogen uptake by the plant may result in toxic effects to the leafhopper. The unusually high nitrogen contents produced in laboratory plants in the current study are probably well above those naturally encountered in the field. However, our results suggest that small to moderate increases in host nitrogen are detected by, and beneficial to, developing *Carneocephala* (see also Rossi and Strong, 1991). Moreover, the two herbaceous species appear to be superior hosts for *Carneocephala*. For instance, nymphs reared on nonfertilized *Borrichia* and *Salicornia* produced insects that were similar in size to field-collected adults (>3 mg for females and >1.4 mg for males) (Rossi, 1991; Rossi and Strong, 1991). Only insects reared on *Distichlis* at the lowest nymphal densities and highest nitrogen levels produced adults similar to field-collected adults.

Recently, Rossi et al. (1994) reported that plant- and parasitoid-mediated mortality also was significantly lower on the two herbaceous species compared to the two grass species. Selective pressures to avoid natural enemies and utilize plants of superior quality appear to be working together to maintain the apparent hierarchy of hosts in *Carneocephala*'s diet. This study supports the contention that *Borrichia* and *Salicornia* are superior hosts for *Carneocephala* and that this leafhopper should utilize these hosts until they become unavailable through winter dieback or until changes in their chemistry make them unsuitable for growth and development.

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STEREOSELECTIVE SYNTHESIS OF DOMINICALURE 1 AND 2: COMPONENTS OF AGGREGATION PHEROMONE FROM MALE LESSER GRAIN BORER *Rhyzopertha dominica* (F.)

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Abstract—Dominicalure 1 (**9a**) and dominicalure 2 (**9b**), were synthesized by esterification of α,β-unsaturated acids **4a** and **4b** with (*S*)-(+)-2-pentanol (**8**). The key step was the asymmetric reduction of 3-penten-2-one (**5**) to give the chiral intermediate **6**, which, upon diimide reduction, DNB derivatization, recrystallization, and hydrolysis, yielded **8** in 63% ee. Acids **4a** and **4b** were prepared in a simple and efficient three-step synthesis with an overall yield of 54% and 62%, respectively, in stereoisomerically pure form.

Key Words—Pheromone, *Rhyzopertha dominica*, Coleoptera, Bostrichidae, dominicalure, (*S*)-(+)-2-pentanol, asymmetric reduction, (*E*)-2-methyl-2-pentenoic acid, (*E*)-2,4-dimethyl-2-pentenoic acid.

INTRODUCTION

The lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), is a worldwide primary pest of stored products. The aggregation pheromone of this beetle was isolated and identified as a mixture of (*S*)-(+)-1-methylbutyl (*E*)-2-methyl-2-pentenoate and (*S*)-(+)-1-methylbutyl (*E*)-2,4-dimethyl-2-pentenoate and named dominicalure 1 and 2, respectively (Williams et al., 1981). Very few syntheses of this pheromone have been reported so far. In one (Williams et al., 1981), glutamic acid was used as a source of chirality giving (*S*)-2-pentanol; the method suffered from low overall yield. In another synthesis (Lin-Yu and Guo-Qiang, 1990), the key step was Sharpless asymmetric epox-

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idation, but surprisingly low values of specific rotation of the final compounds were obtained. Recently, syntheses of dominicalure 1 (Rossi et al., 1993) and dominicalure 2 (Rossi et al., 1992) have been described, using commercially available (*S*)-2-pentanol.

We report here a new synthesis of dominicalure 1 and 2.

METHODS AND MATERIALS

Instrumentation

Gas chromatography analyses were conducted in a Shimazu model GC-14B, equipped with a flame ionization detector and a capillary column β-DEX 110 (30 m × 0.25 mm, Supelco Co.); helium was used as the carrier gas at 130 kPa, split 100:1. NMR (^{1}H and ^{13}C) spectra were obtained with a Varian Gemini-200 on $CDCl_3$ solutions. Infrared spectra were recorded on a Nicolet 510M FT-IR. Optical rotations were measured in a JASCO-DIP-370 polarimeter. Elemental analyses were performed in a Carlo Erba EA1108 Analyzer. Ozone gas was generated from a Fischer Ozon Generator 500M apparatus. For column chromatographic separations, SDS silica gel 230–400 mesh was used.

Reagents

Propanal, 97% (Aldrich) (**1a**); 2-methylpropanal, 98% (Aldrich) (**1b**); 1-chloro-2-butene, >95% (Merck); sodium chlorite, 80% (Aldrich); 3-penten-2-one, 65% (Aldrich) (**5**); 3,5-dinitrobenzoyl chloride, >98% (Fluka); and LAH 1.0 M solution in THF (Aldrich) were the reagents used. Optically pure (*S*)-(−)-binaphthol was prepared according to the Kazlauskas (1990) procedure, mp = 208–210°C, $[\alpha]_D^{23} = -33.5°$ (THF, $c = 1.0$).

Synthesis

*(E)-2-Methyl-2-pentenoic acid (**4a**) (Figure 1).* To a solution of saturated aqueous ammonium chloride/THF (50 ml/10 ml) were added 13.58 g (150 mmol) of 1-chloro-2-butene, 4.35 g (75 mmol) of **1a**, and then 10.32 g (150 mmol) of zinc dust. After stirring the grey suspension overnight at room temperature, a white precipitate of inorganic salts appeared. The suspension was filtered through celite and the liquid extracted with ether (3 × 20 ml). The organic layer was dried and concentrated to give 6.86 g of a colorless liquid (**2a**). This was taken up in dry methanol (120 ml), cooled to −78°C, and a stream of ozone was bubbled through the solution for 4 hr. Then 18 g of Me_2S in 20 ml of MeOH was added; after 10 min the reaction mixture was allowed to warm to room temperature, the solvent was removed, and the residue was dissolved in 100 ml of dichloromethane. An aqueous solution of NaOH (180

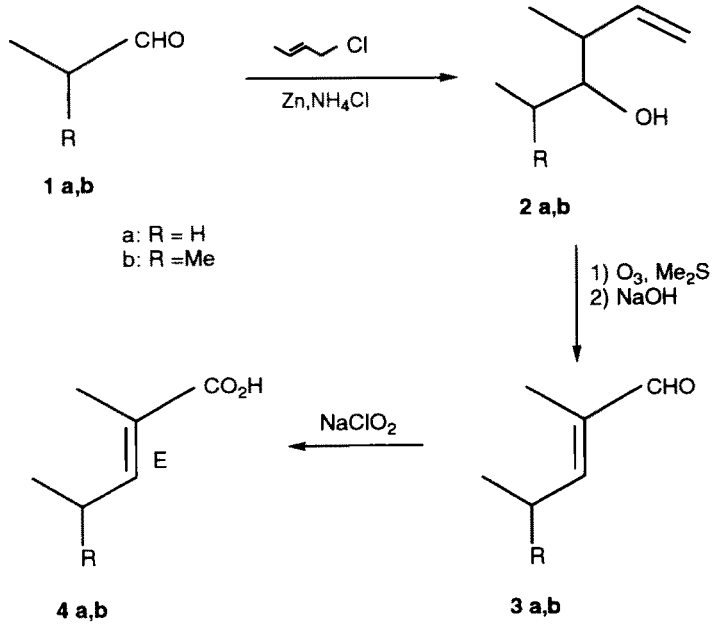


FIG. 1. Synthesis of α,β-unsaturated acids **4a** and **4b**.

ml) was added in small portions and stirring was continued for about 20 min. After extractive work-up, 7 g of a colorless liquid (**3a**) was obtained. Finally, oxidation of aldehyde with sodium chlorite according to Dalcanale and Montanari (1986) afforded 5.8 g of a colorless liquid, which was purified by column chromatography (hexane–ethyl acetate, 5 : 1) to yield 4.62 g of chemically and stereoisomerically pure acid **4a** (54% overall yield from **1a**). IR (neat): 1687 (C=O), 1645 (C=C) cm^{-1}; ^1H NMR: δ 1.02 (t, J = 7.5 Hz, 3H); 1.80 (s, 3H); 2.18 (quintet, J = 7.4 Hz, 2H); 6.87 (tq, J = 7.4 and 1.4 Hz, 1H); 11.70 (br s, 1H); GC: temp. 150°C R_t = 3.65 min (single peak).

(E)-2,4-Dimethyl-2-pentenoic acid (4a) (Figure 1). The procedure described for synthesis of **4a** was used. From 5.4 g of **1b**, 5.9 g of pure acid **4b** (62% overall yield) was obtained. IR (neat): 1687 (C=O), 1645 (C=C) cm^{-1}; ^1H NMR: δ 1.00 (d, J = 6.6 Hz, 6H); 1.81 (s, 3H); 2.57–2.65 (m, 1 H); 6.69 (dq, J = 9.7 and 1.4 Hz, 1H); 11.00 (br s, 1H); GC: temp. 150°C, R_t = 3.70 min (single peak).

(S)-(+)-2-Pentanol 3,5-dinitrobenzoate Ester (7) (Figure 2). A solution of the unsaturated ketone **5** (1.6 g, 18.7 mmol) in THF (15 ml) was added dropwise at −100°C to (*S*)-BINAL-H generated from LAH (56 ml, 56 mmol), ethanol

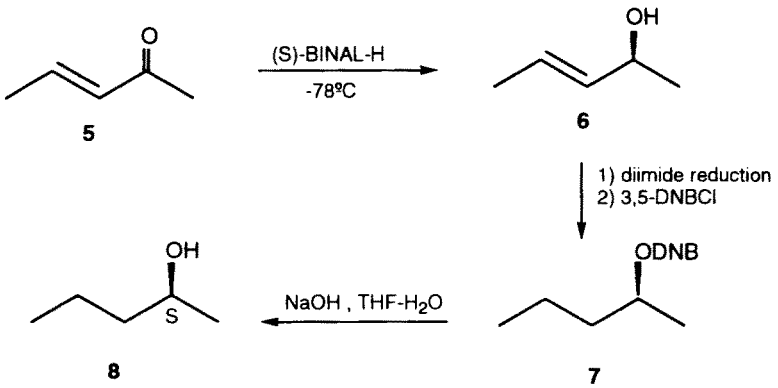


FIG. 2. Synthesis of (*S*)-2-pentanol.

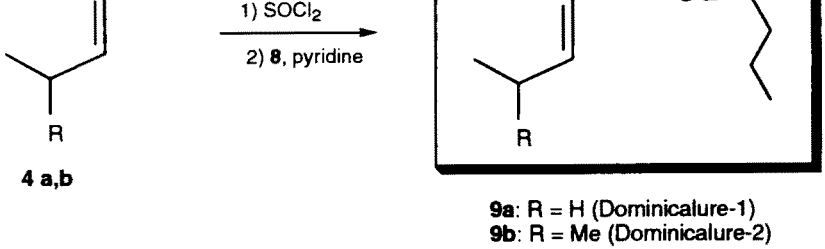
(2 M THF solution, 28.3 ml, 56.6 mmol), and (*S*)-binaphthol (16.2 g, 56.8 mmol). The mixture was stirred for an additional 1 hr at −100°C and then at −78°C for 25 hr. Methanol (3 ml) and water (5 ml) were added, and the mixture was warmed to room temperature. Ether (120 ml) was added, and the solution was stirred for 1 hr and treated with anhydrous magnesium sulfate for 1 hr more. The suspension was filtered through celite, and the filtrate was washed with 1 N HCl (3 × 40 ml) to remove aluminum salts. The organic phase was extracted with 1 N NaOH (3 × 60 ml) and, after neutralization and extractive work-up of the aqueous layer, 13.2 g of (*S*)-binaphthol was recovered. The ethereal phase was concentrated with caution due to the volatility of unsaturated alcohol **6.** For diimide reduction, MeOH (120 ml) was added and then potassium azodicarboxylate (78 g, 400 mmol) and acetic acid (30 ml, 529 mmol) in MeOH (60 ml). After stirring overnight, the yellow color of the solution disappeared. After work-up and careful evaporation of the solvent, the saturated alcohol was converted to its 3,5-dinitrobenzoate derivative by adding dropwise at 0°C a solution of the alcohol in CH_2Cl_2 (20 ml) to a solution of 3,5-dinitrobenzoyl chloride (6.13 g, 26 mmol), DMAP (0.2 g, 1.6 mmol), and Et_3N (7.2 ml, 52 mmol) in CH_2Cl_2 (90 ml). The mixture was stirred at room temperature for 5 hr, and after extractive work-up, 2.9 g of crude **7** was obtained as a brown oil. Purification by column chromatography (hexane–ethyl acetate, 40:1) yielded 2 g of pure **7** as a white solid (43% from **5**). IR (KBr): 1716 (C=O), 1545, 1339 (NO_2) cm^{-1}; ^1H NMR: δ 0.94 (t, J = 7.1 Hz, 3 H); 1.38 (d, J = 6.2 Hz, 3H); 1.45–1.81 (m, 4H); 5.25 (sextet, J = 6.3 Hz, 1H); 9.11–9.19 (m, 3 H); ^{13}C NMR: δ 13.91, 18.76, 20.01, 37.94, 74.27, 122.08, 129.26, 134.42, 148.46, 161.93; $[\alpha]_D^{24}$ = +13.5° (c = 1, $CHCl_3$). Compound **7** (0.7 g) was recrystallized from hexane–ether (20:1) yielding white crystals (0.35 g). Spec-

troscopic data were as above; mp = 85.5–86.5°C; Anal. calc. for $C_{12}H_{14}N_2O_6$: C, 51.06; H, 4.99; N, 9.92; Found: C, 50.84; H, 5.28; N, 9.85; $[\alpha]_D^{24}$ = +20.8° (*c* = 1, $CHCl_3$).

(S)-(+)-2-Pentanol (8) (Figure 2). To a stirred and ice-cooled solution of recrystallized compound **7** (0.35 g) in THF (10 ml) was added dropwise aq. 2 N NaOH (20 ml). After stirring for 5 hr at room temperature followed by extractive work-up, 74 mg (68%) of **8** was obtained (63% ee by GC of its acetate derivative).

Determination of Optical Purity of 8. Alcohol **8** (40 mg) was treated with acetic anhydride (0.5 ml) and pyridine (0.5 ml) and the mixture stirred at room temperature for 4 hr to give the acetate ester. After extractive work-up, the organic phase was analyzed by GC. At 70°C the retention times were 4.88 min [(*S*)-acetate] and 5.54 [(*R*)-acetate].

(S)-(+)-1-Methylbutyl (E)-2-methyl-2-pentenoate (9a) (Figure 3). A solution of compound **4a** (0.08 g, 0.7 mmol) in 5 ml of $CHCl_3$ and thionyl chloride (0.2 ml, 2.6 mmol) was heated to 60°C for 90 min, and the excess thionyl chloride was removed under vacuum. The crude carboxylic acid chloride obtained was treated with a solution of **8** (37 mg, 0.42 mmol) in chloroform (6 ml) and pyridine (0.12 ml, 1.5 mmol) at 50°C for 2 hr and then at room temperature overnight. The mixture was treated with sodium bicarbonate and extracted with ether. The organic phase was washed with brine, dried, and concentrated yielding an oil that was purified by column chromatography using hexane–dichloromethane (5:1) to give **9a** as a colorless liquid with a sweet odor (60 mg, 79%). IR (neat): 1709 (C=O), 1645 (C=C) cm^{-1}; ^1H NMR: δ 0.78 (t, *J* = 7.1 Hz, 3H); 0.91 (t, *J* = 7.5 Hz, 3H); 1.10 (d, *J* = 6.2 Hz, 3H); 1.14–1.65 (m, 4H); 1.68 (s, 3H); 2.05 (quintet, *J* = 7.5 Hz, 2H); 4.82 (sextet, *J* = 6.2 Hz, 1H); 6.58 (tq, *J* = 7.4 and 1.3 Hz, 1H); $[\alpha]_D^{25}$ = +21.5° (*c* = 0.74, ether).



4 a,b

9a: R = H (Dominicalure-1)
9b: R = Me (Dominicalure-2)

FIG. 3. Preparation of dominicalure 1 and 2 from acids **4a** and **4b**.

(S)-(+)-1-Methylbutyl (E)-2,4-dimethyl-2-pentenoate (9b) (Figure 3). The procedure for preparation of **9a** was used. From 37 mg of **8,** 65 mg (76%) of pure **9b** was obtained. IR (neat): 1709 (C=O), 1645 (C=C) cm^{-1}; ^1H NMR: δ 0.9 (t, J = 7 Hz, 3H); 0.99 (d, J = 6.6 Hz, 6H); 1.21 (d, J = 6.3 Hz, 3H); 1.27–1.63 (m, 4H); 1.80 (d, J = 1.5 Hz, 3H); 2.54–2.63 (m, 1H); 4.90 (sextet, J = 6.5 Hz, 1H); 6.52 (dq, J = 9.6 and 1.4 Hz, 1H); $[\alpha]_D^{25}$ = +21.8° (c = 0.84, ether).

RESULTS AND DISCUSSION

One of the most common methods to obtain α,β-unsaturated esters is the Wittig reaction, or any of its modifications, from which a mixture of *E/Z* isomers is always formed (Wadsworth, 1977). The need for stereoisomerically pure *E* acids **4a** and **4b** required an alternative route. These acids have been prepared in stereoisomerically pure form using stereo-defined organometallic compounds, but requiring a lengthy synthesis with only moderate overall yields (Rossi et al., 1992, 1993).

The first step in the synthesis (Figure 1) was the reaction of the allylic zinc reagent with propanal or 2-methylpropanal to give the alkenes **2** in almost quantitative yield and pure enough for the following steps. This is a well-documented process for C—C bond formation (Yamamoto, 1993). The oxidative cleavage of the alkenyl group was then studied in an attempt to directly obtain the acids **4,** but it proved difficult to dehydrate the β-hydroxyacids. So, under reductive ozonolysis, followed by addition of aqueous NaOH solution, the aldehydes **3** were obtained. Finally, oxidation of the aldehyde group with sodium chlorite (Dalcanale and Montanari, 1986), yielded pure acids **4a** and **4b** in an overall yield from **1a** and **1b,** of 54% and 62%, respectively. Capillary GC analysis and NMR spectroscopy of **4a** and **4b** showed that the corresponding *Z* isomers were undetectable. In the course of this work we found it not worthwhile to isolate and purify intermediates **2** and **3** due to their volatility. Spectral properties of analytical samples of these compounds were consistent with their structures.

The key step was the asymmetric synthesis of (*S*)-(+)-2-pentanol, **8** (Figure 2). The known BINAL-H reduction (Noyori, 1984b) was applied to the alkenyl ketone **5** yielding the chiral intermediate **6,** which was not isolated because of its volatility. The progress of the reaction was followed by GC: temperature 60°C, R_t = 5.10 min for **6;** R_t = 4.57 min for **5.** After diimide reduction of **6,** reaction with 3,5-dinitrobenzoyl chloride gave **7** in 43% yield from commercially available **5** (67% with respect to pure ketone). The advantage of isolating **7** is that upon recrystallization, the optical purity of alcohol **8** can be increased. The specific rotation of dinitrobenzoate **7** was +13.5° prior to recrystallization and +20.8° after recrystallization, which corresponds to an increase

in the enantiomeric excess of alcohol **8** from 41% to 63%. This was determined in each case from GC of its acetate derivative. The reduction of the saturated ketone 2-pentanone with (*R*)-BINAL-H under the same conditions gave (*S*)-2-pentanol **8** in only 16% ee; this is in agreement with Noyori's explanation about the effect of electronic as well as steric factors on the direction and extent of the enantioselective reduction with the BINAL-H reagent (Noyori, 1984a).

Dominicalure 1 and 2, **9a** and **9b** (Figure 3) were prepared from acids **4a** and **4b** by esterification through the corresponding acid chloride **8** and pyridine. An excess of alcohol was employed to obtain racemic dominicalure 1 and 2 as reference compounds because inexpensive 2-pentanol was the reagent. However, for the synthesis of the optically active pheromone **9a** and **9b,** an excess of acids **4a** and **4b** was used with comparative yields. The advantage of this procedure is that it is easier to recover unreacted acids **4a** and **4b** than unreacted alcohol **8,** if necessary. Thus obtained **9a** had a specific rotation $[\alpha]_D^{25} = +21.5°$ ($c = 0.74$, ether) [lit. for natural dominicalure 1: $[\alpha]_D^{25} = +32.1°$ ($c = 0.156$, ether) Williams, 1981]. For **9b:** $[\alpha]_D^{25} = +21.8°$ ($c = 0.84$, ether) [lit. for natural dominicalure 2: $[\alpha]_D^{25} = +32.3°$ ($c = 0.777$, ether), Williams, 1981].

In conclusion a simple and efficient three-step synthesis of the stereoisomerically pure acids **4a** and **4b** was developed, which could be applied to the synthesis of other (*E*)-α,β-unsaturated acids. Although there may be better methods of preparing (*S*)-2-pentanol, Noyori's reduction procedure permits the synthesis of both enantiomers and recovery of optically active binaphthol without any racemization. Furthermore, the isolation of dinitrobenzoate **7** is a means of purification and enantiomeric enhancement of chiral alcohol **8,** an otherwise difficult compound to handle.

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TOWARDS A RAPID NEAR-INFRARED TECHNIQUE FOR PREDICTION OF RESISTANCE TO SUGARCANE BORER *Eldana saccharina* WALKER (LEPIDOPTERA: PYRALIDAE) USING STALK SURFACE WAX

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Abstract—Multiple regression predictive models based on data acquired by near-infrared (NIR) spectrophotometry suggest that stalk surface wax components contribute towards resistance to *Eldana saccharina* Walker in sugarcane. At least 35 sugarcane clones of known resistance were required to calibrate a predictive model that accounted for approximately 54% of the variation in resistance to *Eldana*. Wavelengths chosen in multiple regression models suggest that alcohols and carbonyls are important in the wax contribution. Through the use of wax fractionation and gas chromatography, a high alcohol/aldehyde ratio and shorter carbon chain length appears to be associated with resistance. The use of NIR in the screening of wild germplasm and the early screening of breeding material for resistance, without prior knowledge of the biochemical mechanisms involved, is an exciting prospect. However, cause-and-effect relationships remain to be shown.

Key Words—Near infrared, *Eldana saccharina*, sugarcane, host plant resistance, surface wax, herbivory, multiple linear regression, metabolic profiling, Lepidoptera, Pyralidae.

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INTRODUCTION

The stalk borer *Eldana saccharina* Walker is the major pest of sugarcane (*Saccharum* spp. hybrids) in South Africa (Atkinson et al., 1981). Since 1979 variety resistance to the stalk borer has been a selection criterion in the plant breeding program (Bond, 1988). However, field-based selection is largely restricted to one of five selection sites where borer numbers are relatively high, and the effectiveness of the selection program is further limited by the presence of low levels of the pest during years of high rainfall (Anon., 1984). The development of rapid, laboratory-based techniques, applicable to early selection stages when many clones are present, is desirable.

Newly hatched borer larvae spend about one week on the stalk surface before survivors attempt to bore in. Observed differences in sugarcane resistance to borers might therefore be partly explained by biochemical influences of the stalk surface on larval behavior and survival. Surface wax components have been shown to influence the behavior and survival of insect pests on sorghum and maize, two panicoid species related to sugarcane (Woodhead and Taneja, 1987; Yang et al., 1993). Sorghum and maize waxes also have been shown to vary in their component compositions between varieties (Avato et al., 1990a,b).

The technique of component profiling (metabolic profiling), as applied to insect-plant interactions, usually involves the chromatographic separation and quantification of components in a relevant extract followed by implication of individual components in either resistance or susceptibility (Brennan et al., 1992). This can result in a multiple regression model for the prediction of the proportion of resistance conferred by those components. However, chromatographic techniques do not lend themselves to the rapid screening of large numbers of genotypes required in sugarcane plant breeding selection programs.

Organic components can be more rapidly analyzed in an unseparated mixture by measuring spectral absorbances in the near infrared (NIR) (Osborne and Fern, 1986). Spectra of complex organic materials are composed of overlapping spectra for all of the individual components. Complex NIR data can, if necessary, be effectively analyzed by powerful multivariate chemometric packages for the prediction of components (Martens and Naes, 1984). Frequently, however, stepwise multiple regression is found to be adequate.

Given the solubility of sugarcane wax in NIR transparent carbon tetrachloride, and the ability of NIR to analyze mixtures, we decided to explore this technique as a means to profile unknown sugarcane wax components in relation to known larval survival ratings for the stalk borer. To our knowledge, NIR has never been used to quantify insect resistance in any crop. If this proved possible, NIR technology would greatly expedite sugarcane germplasm development, allowing the early identification of clones with a measure of stalk borer resistance.

The objectives of this study were: (1) to determine whether sugarcane wax NIR data could be correlated with borer resistance, (2) to gain some insight from the NIR data as to the identity of any likely active components, and (3) if successful in predicting borer resistance, to develop a rapid NIR technique for the prediction of wax-based resistance in unclassified clones.

METHODS AND MATERIALS

Wax Preparation for NIR and GC Analyses. Lengths of stalk from the base to the natural breaking point (from a time when 1 m plus of stalk had accumulated) were scraped with razor blades. Five stalks per clone were sampled yielding 100–200 mg of crude wax. Scraped crude wax (100 mg) was dissolved in 10 ml of helium-degassed carbon tetrachloride under nitrogen in an oven at 80°C for 10 min. This was then filtered through glass wool. Samples for GC contained 0.5 mg/ml of tricosane ($C_{23}H_{48}$) as an internal standard.

NIR Feasibility Study—Filter-Based Spectrophotometer. Series of commercial varieties from several seedcane nurseries and of increasing cane age were sampled in an initial feasibility study. A model 400 InfraAlyzer (Technicon Industrial Systems, New York, New York), operating 19 different optical filters with wavelengths from 1445 to 2348 nm, was used to acquire transflectance values as log 1/reflectance (log $1/R$) for wax dissolved in carbon tetrachloride. Samples (180 μl) were placed on a transflectance cell under a glass slide and each was read three times by the InfraAlyzer 400. Resistance ratings based on survival of borer larvae were entered into the accompanying InfraAlyzer calibration software (IACAL) as knowns for each clone. Stepwise linear multiple regression on log $1/R$ filter readings against resistance ratings was performed.

Determination of Resistance Ratings. Cane was grown in buckets for nine months before being stressed through reduced watering followed by inoculation with stalk borer eggs (Nuss and Atkinson, 1983). Assessment of resistance trials for larval survival, larval mass, and percent internodes damaged was made 500 day-degrees (with a developmental threshold of 10°C) after inoculation. Each clone was rated by each of the above measures and an average rating was obtained.

In this paper a rating based on larval survival alone was used, since final larval numbers appear to be decided during the approximate one-week period larvae spend on the stalk surface before stalk entry (K. J. Nuss, personal communication). Survival ratings are expressed as low 1, 2, and 3 (resistant); intermediate 4, 5, and 6; and high 7, 8, and 9 (susceptible), based on comparison with five standard varieties.

Calibration of Scanning NIR Spectrophotometer. An NIRSystems 5000 scanning monochromator (NIRSystems, Silver Spring, Maryland) was used to

acquire transmission spectra (1-cm light path) with near-infrared radiation from 1100 to 2500 nm for waxes from an expanded calibration set of 40 clones including both commercial varieties and unreleased late selection stage clones. Borer larval survival ratings were entered as knowns into the accompanying NIRSystems spectral analysis software (NSAS). Stepwise linear multiple regression was performed on the second derivative of log $1/R$ spectra against survival ratings.

Determination of Wax Load and Composition. Crude scraped waxes were obtained from commercial varieties N20 and N14 at 8 and 12 months of age. Stalk surface area and mass of crude wax were recorded. Dissolved crude waxes were filtered, and after drying, carbon tetrachloride insoluble and soluble fractions were determined gravimetrically. The soluble wax was further fractionated on a silicic acid column eluted with cyclohexane followed by chlorofrom and then chloroform–ethyl acetate (95:5 v/v) and finally ethyl acetate. Fractions were roughly designated as follows: 1, alkanes; 2, esters and aldehydes (carbonyls); 3, alcohols; and 4, acids. Quantities were determined gravimetrically and fractions were analyzed by GC.

Gas Chromatography. An OV-5 30-m × 0.32-mm ID capillary column was used. Approximately 0.4 μl of 10 mg/ml whole wax solutions or fractions thereof were injected without splitting onto a cold column (50°C for 1 min). The column was then heated to 200°C at 10°C/min and held for 1 min. The main separation occurred during the temperature rise at 5°C/min to 310°C. Injector and detector (FID) temperatures were 310°C. The carrier gas was nitrogen at 1.6 ml/min.

RESULTS

Results from an initial feasibility study suggest that chemical differences exist in sugarcane wax that can be correlated with resistance or susceptibility to the stalk borer. Wavelengths chosen for each regression (material from different sites and of different ages) were fairly stable, indicating that the same wax components were implicated (Table 1). In each model, 1982 nm (1940 nm on one occasion) was chosen first as the most highly correlatable single wavelength. As a general trend, it appeared that with increasing cane age the correlation coefficient declined, the y intercept (on the predicted rating axis) increased, and the slope of the regression line decreased.

Results from the feasibility study suggested that progress might be made with an expanded calibration set. Forty clones of known larval survival ratings were sampled at 8 (Figure 1), 10, and 12 months of age for use in conjunction with an NIRSystems 5000 scanning spectrophotometer. The results obtained suggest that variations in wax characteristics might account for around 53.5%

TABLE 1. INITIAL FEASIBILITY STUDY CALIBRATIONS WITH FILTER-BASED NIR INSTRUMENT (log $1/R$)

No. of varieties	13	8	8	9	13	16
Cane age (months)	8	8	9	10	11	12
Wavelength (nm):	(−)1982	(−)1982	(−)1982	(−)1982	(−)1940	(−)1982
(coeff. + or −)	(+)2139	(+)2139	(+)2139	(+)2139	(+)2100	(+)2139
	(+)1818	(+)1778	(+)1778	(+)1818	(+)1734	(+)1818
y intercept	0.8	1.2	1.1	3.5	3.7	3.4
Gradient	0.85	0.72	0.77	0.55	0.41	0.48
r^2	0.89	0.73	0.86	0.78	0.48	0.57
Multiples of $P = 0.05$ F requirement	6.29	0.54	1.89	1.09	0.72	1.52

of the variation in larval survival ratings (Table 2). Again, the correlation between predicted and known ratings declined with increasing cane age.

Ideally a calibration set should be large enough that all the variation in the population has been sampled, i.e., the correlation coefficient reaches a plateau and the F statistic retains significance. Figure 2 shows that for an enlarging model, both the correlation coefficient and wavelength selection began to stabilize when more than 30 clones were used in the calibration. Since only 40 clones of known larval survival ratings were available, neither further extension of the calibration set nor a blind validation trial were possible.

Wavelengths in the region of 1940–1945 nm were the most highly correlated individual wavelengths throughout the clone addition exercise. According to spectra–structure correlation charts, such as that of Goddu and Delker (1960),

TABLE 2. EXPANDED CALIBRATIONS WITH SCANNING NIR INSTRUMENT; 40 CLONES[a]

	Age of cane		
	8 months	10 months	12 months
Wavelength (nm):	(−) 1942	(+) 1846	(−) 1490
(Coeff: + or −)	(+) 2072	(+) 2426	(+) 2424
	(−) 1600	(+) 2192	(−) 2470
r^2	0.535	0.378	0.315
Multiples of $P = 0.05$ F requirement	4.81	2.53	1.92
SE of the estimate	1.78	2.07	2.25

[a]Second derivative of log $1/R$: segment 10, gap 8.

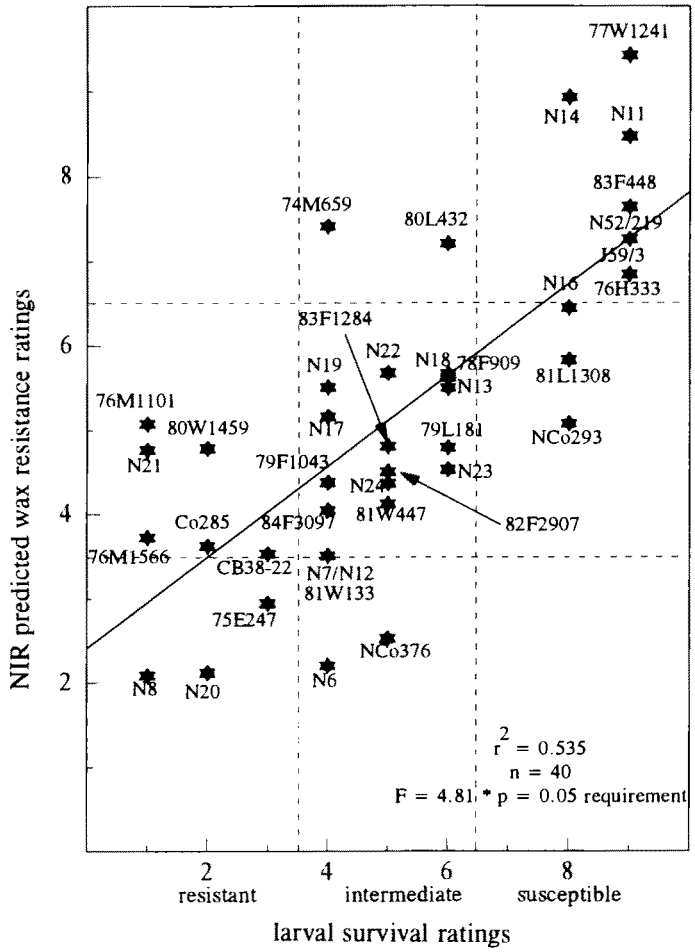

FIG. 1. Relationship between larval survival ratings and NIR wax predicted ratings for 8-month-old cane (40 clones).

wavelengths selected by both the IACAL and NSAS softwares suggest that alcohols and carbonyls might be important in the interaction between sugarcane and the stalk borer. In waxes, 1940–1945 nm might be characteristic of alcohols (−OH) or carbonyls (−C=O). Wavelengths in the region of 2070 nm are also characteristic of alcohols, as well as of hydroperoxides. Hydroperoxides are known to be present in sugarcane wax (Lamberton and Redcliffe, 1960).

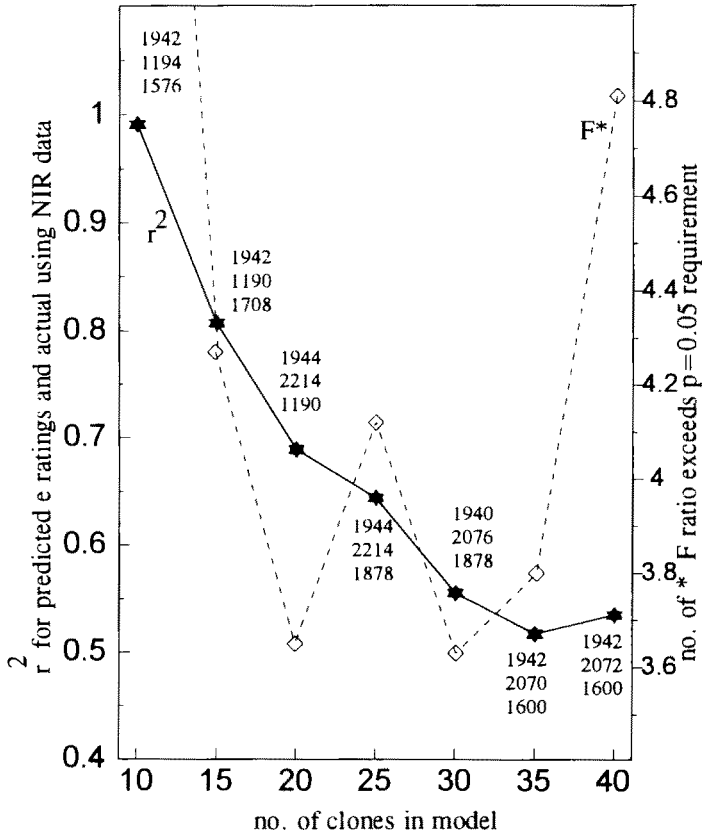

FIG. 2. Number of clones required to sample NIR variation in wax resistance (8-month-old cane).

GC wax chromatograms of resistant N20 and susceptible N14 at 8 months highlight the extremes of GC profile type among commercial varieties (Figure 3). These two varieties also differed greatly in NIR prediction.

Fractionation of wax and analysis by GC revealed that peaks 4, 6, and 8 were in the alcohol fraction while peaks 3, 5, and 7 were in the ester/aldehyde fraction. Peaks 3, 5, and 7 were independently identified by GC–mass spectroscopy (GC-MS) as the C_{26}, C_{28}, and C_{30} aldehydes, respectively, while peak 6 was identified as the C_{28} alcohol. The C_{28} aldehyde and alcohol are known to be components of sugarcane stalk wax (Tulloch, 1976). Due to their frac-

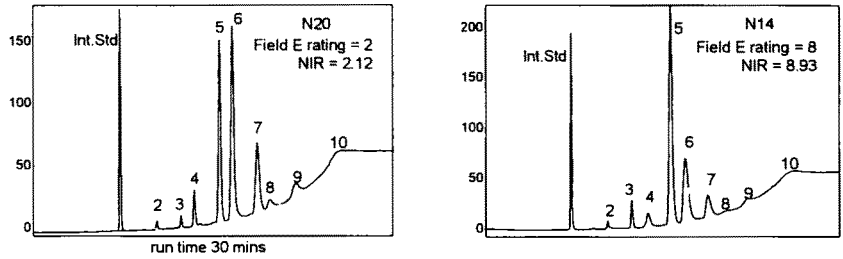

FIG. 3. Gas chromatograms of wax from resistant (N20) and susceptible (N14) commercial clones.

tionation properties, peaks 4 and 8 were considered to be the C_{26} and C_{30} alcohols, although they were not positively identified by GC-MS.

Fractionation of waxes from resistant and susceptible varieties suggests that the alcohol fraction decreases as a proportion of the total with both susceptibility and age. In addition, wax load decreased with age while nonsoluble contaminants (mostly dirt and fungal hyphae) increased (Table 3).

In order to further implicate GC peaks in resistance or susceptibility, peak areas relative to the internal standard were entered into the IACAL software. Using larval survival ratings as knowns, peaks were associated with either resistance (negative coefficients) or susceptibility (positive coefficients). Peaks were similarly associated with 8-month NIR predicted ratings.

TABLE 3. LOAD AND FRACTIONATION OF CRUDE SCRAPED WAX FROM VARIETIES N20 (RESISTANT) AND N14 (SUSCEPTIBLE) AT 8 AND 12 MONTHS OF AGE

Variety and age	Nonsoluble load (mg/100 cm^{-2})	Soluble load (mg/100 cm^{-2}) (% of 8 month total)	Alkane fraction (%)	Carbonyl fraction (%)	Alcohol fraction (%)	Acid fraction (%)	Crude wax in peaks 3–8 (%)[a]
N20 (8 months)	0.5	8.9 (95)	9	43	41	7	29.7
N14 (8 months)	0.3	8.3 (97)	7	57	27	9	19.1
N20 (12 months)	1.2	6.6 (70)	7	52	33	8	22.3
N14 (12 months)	2.3	5.4 (63)	3	60	26	11	18.6

[a]Calculated relative to internal standard tricosane ($C_{23}H_{48}$).

TABLE 4. GC PEAK MODELS FOR THE PREDICTION OF LARVAL SURVIVAL RATINGS AND 8 MONTH SCANNING NIR RESULTS

	Model for			
	Larval survival ratings—40 clones		NIR results—40 clones	
	Coefficient	*t* test	Coefficient	*t* test
Peak	Offset 3.570		Offset 4.455	
3	−0.577	0.74	−0.198	0.47
4	−0.322	0.43	−0.861	2.13
5	+0.318	1.92	+0.251	2.81
6	−0.551	1.80	+0.488	2.96
7	+0.554	0.96	+0.557	1.79
8	+1.132	0.83	+0.721	0.99
Multiples of $P = 0.05$ F requirement	1.05		3.87	
Correlation coefficient (r^2)	0.315		0.628	

The C_{28} (peak 5) and C_{30} (peak 7) aldehydes were associated with NIR predicted susceptibility and known susceptibility (positive coefficients). The C_{26} aldehyde and alcohol and the C_{28} alcohol were associated with resistance while the C_{30} alcohol appeared to be associated with susceptibility (Table 4). It is therefore possible that the high alcohol/aldehyde ratio and shorter chain length might be involved in resistance to the stalk borer. GC peak models accounted for 31.5% and 62.8% of the variation in known larval survival ratings and NIR predited ratings, respectively (Table 4), while the NIR model for the prediction of larval survival ratings accounts for 53.5%. This suggests that the NIR technique profiles a greater proportion of the total wax than the GC technique. Evidently not all of the dissolved wax emerges from the GC column (Table 3).

The GC profiles of three *S. spontaneum* clones can be compared with those of three *S. officinarum* clones (Figures 4 and 5). These data revealed that *S. spontaneum* was dominated by shorter chain length C_{26} rather than C_{28} aldehydes and alcohols, while *S. officinarum* wax resembled that of hybrids.

DISCUSSION

It is not claimed that the relationships between NIR- and GC-determined wax components and resistance or susceptibility to the stalk borer represent cause and effect. Although considerably more clones are desirable in a calibration set, the 40-clone (8-month) NIR model produced does appear to have predictive

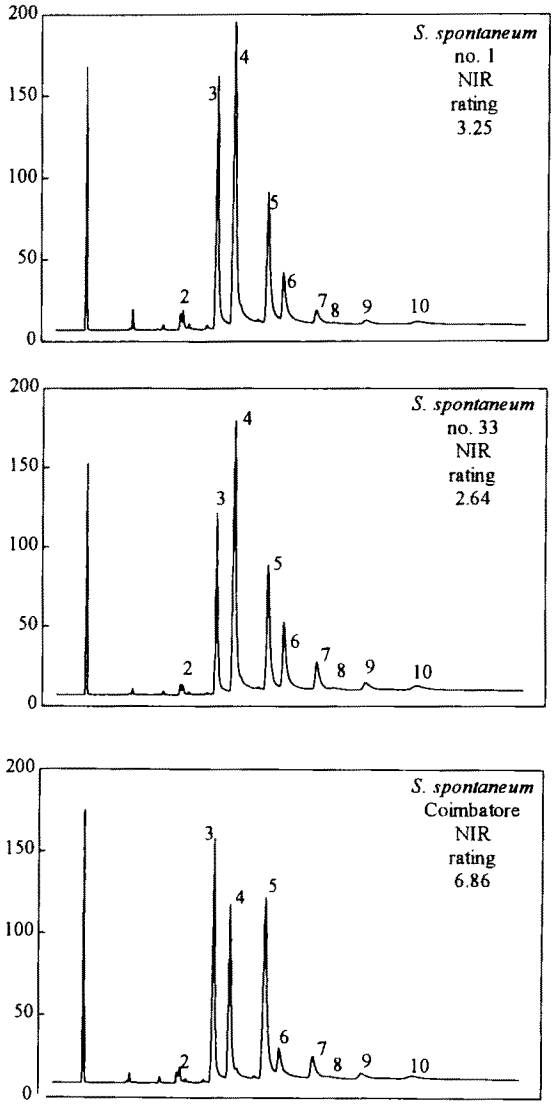

FIG. 4. GC wax profiles for three *S. spontaneum* clones.

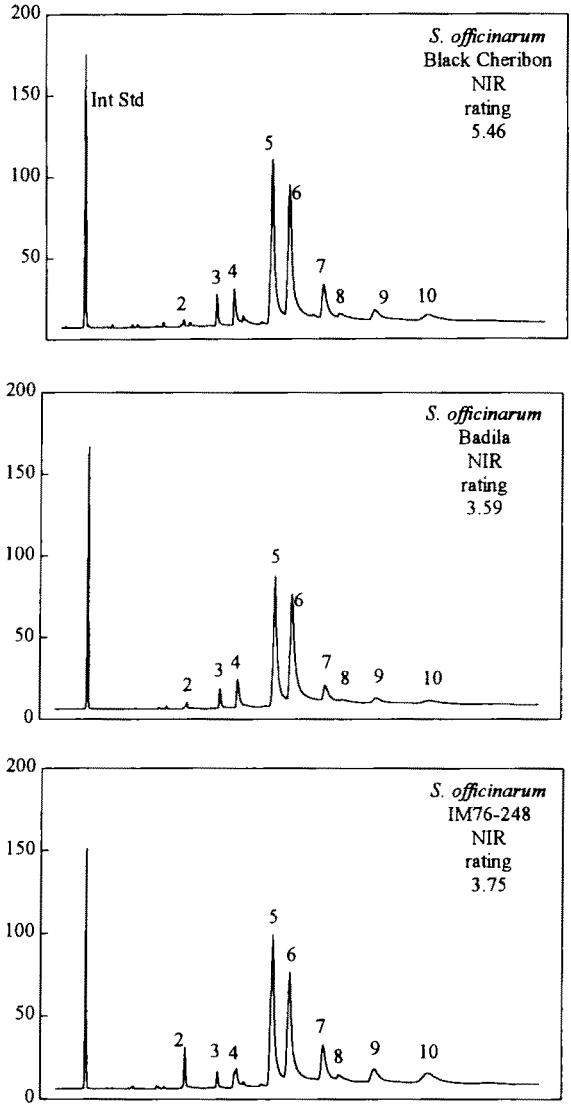

FIG. 5. GC wax profiles for three *S. officinarum* clones.

value. If clones predicted to be susceptible were discarded from a selection program, only 4% of resistant clones would be lost based on a correlation coefficient in the region of 0.53 (Schenk and Westerhaus, 1993). Since variation in wax composition explains 53.5% of variation in larval survival, such clones could be resistant for reasons other than wax composition.

Again based on a correlation coefficient of 0.53, approximately 60% of resistant or susceptible clones should be correctly classified. For susceptible clones in the calibration set, six of nine (66%) were predicted correctly; however, for resistant clones, only 33% were predicted correctly, suggesting a skew (Figure 1). Despite this, no resistant clones were predicted to be susceptible and vice versa.

Decreasing wax load, alcohol content, and NIR model correlation coefficients with increasing cane age all suggest that the wax contribution towards resistance might decline in older cane. Weathering and microbial action are probably the main causes of wax disappearance and degradation (Kolattukudy, 1980). The production of wax alcohols in maize has been shown to decline with increasing plant maturity (Avato et al., 1990b). It is well known that mature cane is more easily infested by the stalk borer than immature cane (Carnegie and Smaill, 1980).

These results suggest that wax alcohols might be associated with resistance while aldehydes appear associated with susceptibility. The indication that shorter chain length is also associated with resistance suggests that additional resistance might be obtained through further introgression of selected *S. spontaneum* germplasm.

S. spontaneum has already played an important role in the development of modern varieties by contributing many desirable characteristics, including disease and drought resistance (Roach, 1984). Modern varieties have a narrow genetic base; however, their genealogies can be traced to two *S. spontaneum* clones (Glagah and Coimbatore) and between 9 and 11 clones of other *Saccharum* species, mostly *S. officinarum* (Arceneaux, 1965). *S. spontaneum* Coimbatore has a susceptible NIR wax prediction and also a more susceptible GC profile when compared with two other unutilized *S. spontaneum* clones. Coimbatore would probably not have been selected for use as an ancestor of modern hybrids if greater resistance to the stalk borer had been the reason for the introgression. Glagah was not available for analysis.

Cause and effect as well as validation of the NIR method remain to be shown. To show cause and effect, experiments designed to demonstrate behavioral or feeding responses to waxes with variant NIR predictions are in progress. To validate the NIR method, sets of unclassified clones are being predicted and their larval survival ratings are being determined by exposure to the insect over several years. These clones can then be added to the calibration set.

NIR offers the potential of a rapid technique for the selection of borer resistant genotypes. Wax scraping is not destructive of clonal seed material and hundreds of clones can be predicted in one day. The use of fiberoptics in conjunction with hand-held NIR instruments for analysis of the stalk surface in situ is a possibility for the future.

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ROLE OF CUTICULAR HYDROCARBONS OF APHID PARASITOIDS IN THEIR RELATIONSHIP TO APHID-ATTENDING ANTS

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Abstract—*Lysiphlebus cardui*, the dominant aphidiid parasitoid of the black bean aphid, *Aphis fabae cirsiiacanthoidis* (Afc), on creeping thistle, is able to forage in ant-attended aphid colonies without being attacked by ants. Several behavioral observations and experimental studies led to the hypothesis that *L. cardui* mimics the cuticular hydrocarbon profile of its host aphid. Chemical analysis of the cuticular extracts revealed that both *L. cardui* and Afc exclusively possess saturated hydrocarbons: *n*-alkanes, monomethyl (MMA), dimethyl (DMA), and trimethyl alkanes (TMA). Comparison of the hydrocarbon profiles of parasitoid and aphid showed great qualitative resemblance between parasitoid and host: *L. cardui* possesses almost all host-specific compounds in addition to species-specific hydrocarbons of mainly higher molecular weight ($>C_{30}$). However, there is a lesser quantitative correspondence between parasitoid and host aphid. Furthermore, we analyzed the cuticular hydrocarbon profile of another parasitoid of Afc, *Trioxys angelicae*. This aphidiid species is vigorously attacked and finally killed by honeydew-collecting ants when encountered in aphid colonies. Its cuticular hydrocarbon profile is characterized by the presence of large amounts of (*Z*)-11-alkenes of chain lenghts C_{27}, C_{29}, C_{31}, and C_{33}, in addition to alkanes and presumably trienes. The role of the unsaturated hydrocarbons on *T. angelicae* as recognition cues for aphid-attending ants is discussed.

Key Words—*Aphis fabae cirsiiacanthoidis*, Aphididae, *Lysiphlebus cardui*, *Trioxys angelicae*, Aphidiidae, parasitoids, ant–parasitoid relationship, cuticular hydrocarbons, chemical mimicry.

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INTRODUCTION

Cuticular surface lipids serve a variety of physiological and behavioral functions in insects (Blomquist and Dillwith, 1985; Jackson and Blomquist, 1976; Lockey, 1988). In recent years aspects of chemical communication via surface lipids have been increasingly investigated. Cuticular hydrocarbons have been shown to play a role as sex pheromones in numerous insect species (for examples, see review of Blomquist et al., 1993), or as nestmate recognition cues in social insects (see Howard, 1993, and references therein). In addition, they also function in chemical mimicry systems. Numerous insect species that integrate into colonies of social insects possess the same cuticular hydrocarbon profile as their host (see reviews of Stowe, 1988; Dettner and Liepert, 1994).

Chemical mimicry was suggested to be involved in the aphid–ant–parasitoid system of the black bean aphid on creeping thistle, *Aphis fabae cirsiiacanthoidis* Schrank (Afc), the ant species *Lasius niger* L., and the aphidiid wasp *Lysiphlebus cardui* (Marshall) (Völkl and Mackauer, 1993). *L. cardui* was supposed to mimic the cuticular hydrocarbon profile of its host aphid. This assumption was based on the observation that *L. cardui* was able to forage in Afc colonies without being attacked by attending ants. In contrast, a second aphidiid species that parasitizes Afc on thistle, *Trioxys angelicae* (Haliday), is rapidly recognized and vigorously attacked by ants when foraging in aphid colonies (Völkl and Mackauer, 1993). This different ant response depends on the cuticular signature of the parasitoid (Liepert and Dettner, 1993). When hexane-washed dead individuals of *L. cardui* were treated with the hexane extract of *T. angelicae* and offered in attended aphid colonies, the ants rapidly seized the parasitoid and transported it off the plant. In contrast, hexane-washed dead *T. angelicae* with the cuticular hexane extract of *L. cardui* did not induce ant attacks.

The purpose of the present study was to determine the cuticular hydrocarbon profiles of both parasitoids and the host aphid. If chemical mimicry is involved in the case of *L. cardui*, then we expect a resemblance of cuticular hydrocarbon profiles between parasitoid and host that makes it impossible for the ants to discriminate between aphid and *L. cardui*. Furthermore, we were interested in which cuticular components of *T. angelicae* might be responsible for triggering ant aggression.

METHODS AND MATERIALS

Insect Material. Aphids and parasitoids were obtained as described in Liepert and Dettner (1993). Neither parasitoid species had contact with the aphid host or its host plant. For the extraction of the cuticular lipids only female

parasitoids (30–40 individuals of each species) were used because they come into contact with aphid-attending ants when searching for hosts in which to oviposit. Aphids of the first to fourth instar (about 200 individuals) were extracted with hexane as these instars are the preferred hosts for both *L. cardui* and *T. angelicae* (Völkl, 1990).

Chemical Analysis. Cuticular hydrocarbons were recovered by extracting the insects twice in hexane for 60 sec at room temperature. The hexane extracts were combined and the solvent was evaporated under a gentle stream of nitrogen. The cuticular extracts were redissolved in 50 μl of hexane. To separate the hydrocarbon fraction from other lipid classes, the cuticular extract was applied to a column (5 cm $\times$ 0.5 cm ID) of silica gel 60 (70–230 mesh, Merck). Hydrocarbons were eluted with 4 ml hexane. Column chromatography was only applied to the aphid extract, since the gas chromatographic analysis of the cuticular extract of *L. cardui* exclusively yielded hydrocarbons. In the case of *T. angelicae*, insufficient sample was available for column chromatography; therefore, the cuticular lipid profile of the parasitoid is presented.

Gas Chromatography (GC) was carried out on a Carlo Erba GC 6000 Vega Series 2 equipped with a split-splitless injector and a flame ionization detector. A fused silica capillary column (30 m $\times$ 0.32 mm) with a chemically bonded DB-1 stationary phase of 0.10-μm film thickness was used for the analysis. Helium was the carrier gas with a flow rate of 1.5 ml/min. The oven temperature was kept at 180°C for 5 min, increased to 250°C at a rate of 15°C/min, then further increased at 6°C/min to the final temperature of 325°C, which was kept constant for 10 min. For diagnostic purposes an *n*-alkane standard from C_{20} to C_{36} was injected.

GC-MS was performed on a Finnigan MAT 95 connected with a Varian 3700 chromatograph that was equipped with a fused silica capillary column (DB-1, 30 m $\times$ 0.3 mm, FD = 0.25 μm). Helium was used as carrier gas. The oven temperature was programmed from 180°C to 310°C at a rate of 3°C/min. The final temperature was kept constant for 20 min. Mass spectra were obtained at an electron energy of 70 eV.

Substances were identified by their characteristic mass spectral fragmentation patterns and retention times. The mass spectra of some compounds were compared to spectra published in the National Bureau of Standards Mass Spectra Library. The branching positions of methyl-substituted hydrocarbons were determined by the mass ions that were formed by the α-cleavage at the branch point (McCarthy et al., 1968). The localization of double-bond positions in unsaturated lipids was determined by GC-MS analysis of dimethyl disulfide (DMDS) derivatives (Dunkelblum et al., 1985). Double-bond configuration was investigated by GC-FTIR (Bruker analytische Messtechnik GmbH, Karlsruhe, Germany).

RESULTS

Combined GC-MS analysis of the cuticular hydrocarbons of the aphid Afc and the aphidiid species *L. cardui* and *T. angelicae* resulted in the identification of *n*-alkanes, branched alkanes, and unsaturated hydrocarbons (Table 1). The species-specific capillary gas chromatograms obtained from the respective hexane extracts are shown in Figure 1. The numbers given to the peaks in the chromatograms refer to Table 2, where the identification of the signals and the relative mean abundance of each component are summarized.

Normal alkanes dominated in all three species (Table 1). They accounted for almost 70% of the total peak area (TPA) in the aphid Afc and around 50% in the parasitoids. Their chain lengths ranged from C_{23} to C_{35} with the odd-numbered carbon backbone being prevalent (Table 2). Pentacosane (peak 6) was the major component on the aphid and on *T. angelicae*, followed by heptacosane (peak 14) and nonacosane (peak 25) in decreasing quantities; in Afc pentacosane accounted for almost 50% of the TPA. *L. cardui* was characterized by the dominance of *n*-alkanes of higher molecular weight, i.e., nonacosane (peak 25) and hentriacontane (peak 37).

Methyl-substituted components could be found in all three species (Table 1). The parasitoid *T. angelicae* only possessed monomethyl alkanes (MMA) in the range between C_{23} and C_{31}; both terminally branched MMA, i.e., MMA with the methyl group at position 2 and 3, respectively, and internally branched MMA were common on the cuticle of *T. angelicae*. Quantitatively, MMA played a minor role in *T. angelicae* (Table 1). In contrast, Afc and *L. cardui* were characterized by a variety of methyl alkanes. MMA ranged from C_{23} to C_{33} on the aphid and from C_{23} to C_{35} on *L. cardui* (Table 2). This substance

TABLE 1. PROPORTIONS OF DIFFERENT CLASSES OF HYDROCARBONS OF *Aphis fabae cirsiiacanthoidis* (AFC), *Lysiphlebus cardui* AND *Trioxys angelicae*[a]

	Total peak area (mean %)		
Class of compounds[b]	Afc	*L. cardui*	*T. angelicae*
n-Alkanes	69.8	53.6	45.1
MMA	24.5	20.8	9.2
DMA	3.8	21.4	
TMA	Tr[c]	4.2	
Alkenes			44.9

[a] Five aliquots of each extract were injected into the GC.
[b] MMA = monomethylalkanes, DMA = dimethylalkanes, TMA = trimethylalkanes, alkenes = monoenes and trienes.
[c] Tr = trace (<0.2% of the total peak area).

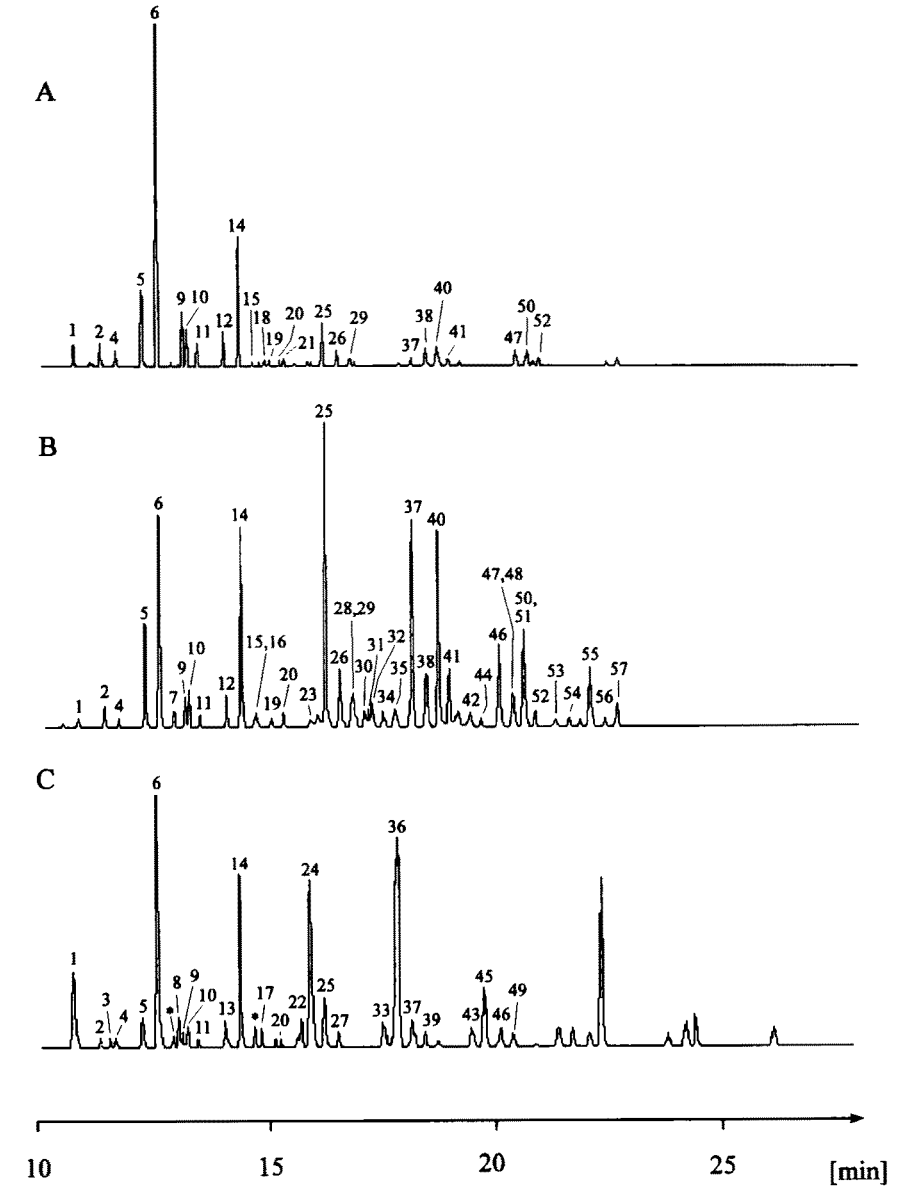

FIG. 1. Gas chromatograms of cuticular hydrocarbons of *A. fabae cirsiiacanthoidis* (A), *L. cardui* (B), and *T. angelicae* (C). Peaks marked by an asterisk indicate methyl alcohols, unmarked peaks in the high molecular mass range indicate polar lipid compounds in the extract of *T. angelicae*. Peaks are listed in Table 2.

TABLE 2. CUTICULAR HYDROCARBONS FROM *Aphis fabae cirsiiacanthoidis* (Afc), *Lysiphlebus cardui* AND *Trioxys angelicae*[a]

Peak	Substance	Total peak area (mean % ± SD)[a]		
		Afc	*L. cardui*	*T. angelicae*
1	n-C_{23}	1.82 (0.37)	0.39 (0.14)	5.66 (0.22)
2	2-MeC_{23}	1.74 (0.29)	0.85 (0.10)	Tr[b]
3	3-MeC_{23}			Tr
4	n-C_{24}	1.20 (0.10)	0.28 (0.11)	0.36 (0.04)
5	2-MeC_{24}	7.21 (0.47)	4.38 (0.44)	2.08 (0.12)
6	n-C_{25}	48.61 (3.50)	11.01 (1.25)	20.54 (0.37)
7	11-MeC_{25}		0.83 (0.10)	
8	5-MeC_{25}			1.71 (0.04)
9	2-MeC_{25}	4.50 (0.29)	1.19 (0.16)	0.51 (0.04)
10	3-MeC_{25}	3.57 (0.08)	1.33 (0.13)	1.00 (0.05)
11	n-C_{26}	1.93 (0.11)	0.47 (0.08)	0.34 (0.02)
12	2-MeC_{26}	2.83 (0.21)	1.41 (0.19)	
13	(Z)-11-C_{27}			2.15 (0.06)
14	n-C_{27}	11.64 (0.57)	8.18 (0.42)	10.93 (0.19)
15	11-MeC_{27}	Tr	0.40 (0.15)	
16	13-MeC_{27}			
17	5-MeC_{27}			1.00 (0.05)
18	2-MeC_{27}	0.20 (0.19)		
19	3-MeC_{27}	0.39 (0.22)	0.21 (0.21)	
20	n-C_{28}	0.24 (0.22)	0.41 (0.05)	Tr
21	unknown	0.68 (0.14)		
22	nonacosatriene			2.05 (0.65)
23	2-MeC_{28}		Tr	
24	(Z)-11-C_{29}			18.01 (0.31)
25	n-C_{29}	4.09 (0.49)	14.34 (1.56)	3.60 (0.14)
26	11-MeC_{29}	0.95 (0.59)	3.03 (0.24)	
27	13-MeC_{29}			1.09 (0.06)
28	9,13-diMeC_{29}	0.24 (0.30)	2.01 (0.75)	
29	11,15-diMeC_{29}			
30	9,13,17-triMeC_{29}		0.65 (0.16)	
31	n-C_{30}		0.28 (0.08)	
32	unknown		0.72 (0.10)	
33	hentriacontatriene			1.24 (0.57)
34	12-MeC_{30}		0.41 (0.17)	
35	10,14-diMeC_{30}		0.92 (0.15)	
36	(Z)-11-C_{31}			16.26 (1.26)
37	n-C_{31}	0.26 (0.36)	11.17 (2.08)	2.24 (0.18)
38	11-MeC_{31}	1.74 (1.01)	3.20 (0.26)	
39	13-MeC_{31}			1.16 (0.06)
40	11,15-diMeC_{31}	2.15 (0.95)	10.20 (1.01)	
41	11,15,19-triMeC_{31}	Tr	2.57 (0.27)	
42	X-MeC_{32}		0.33 (0.14)	

TABLE 2. Continued.

Peak	Substance	Total peak area (mean % ± SD)[a]		
		Afc	*L. cardui*	*T. angelicae*
43	tritriacontatriene			0.44 (0.98)
44	12,16-diMeC$_{32}$		0.59 (0.09)	
45	(Z)-11-C$_{33}$			4.70 (0.33)
46	*n*-C$_{33}$		4.44 (0.95)	1.47 (1.12)
47	11-MeC$_{33}$	1.33 (0.77)	1.50 (0.12)	
48	13-MeC$_{33}$			
49	15-MeC$_{33}$			0.66 (0.07)
50	11,15-diMeC$_{33}$	1.40 (0.34)	5.53 (0.54)	
51	13,17-diMeC$_{33}$			
52	11,15,19-triMeC$_{33}$	Tr	1.01 (0.14)	
53	X-MeC$_{34}$		Tr	
54	12,16-diMeC$_{34}$		0.59 (0.27)	
55	*n*-C$_{35}$		2.44 (0.59)	
56	13-MeC$_{35}$		0.64 (0.14)	
57	13,17-diMeC$_{35}$		1.48 (0.18)	

[a] Five aliquots of each extract were injected into the GC.
[b] Tr = trace (<0.2% of the total peak area).

class accounted for more than 20% of the TPA in both species (Table 1). Furthermore, polysubstituted methyl alkanes were also present. The chain length distribution ranged from C_{29} to C_{35}. The aphid possessed three dimethyl alkanes (DMA) (peaks 29, 40, and 50) with odd carbon numbers. *L. cardui* was characterized by a broader range of DMA; in addition, on the parasitoid DMA of even carbon numbers (peaks 35, 44, and 54) were detected. The relative mean abundance of the DMA on *L. cardui* was more than 21% of the TPA (Table 1). In the aphid this substance group was of minor importance quantitatively. Relatively small amounts of trimethyl alkanes (TMA) in the chain length range of C_{29}–C_{33} could be detected in the extract of *L. cardui* (peaks 30, 41, and 52). The aphid possessed two hydrocarbon components that eluted at the same retention times as 11,15,19-triMeC$_{31}$ (peak 41) and 11,15,19-triMeC$_{33}$ (peak 52) present in the extract of *L. cardui*, but we could not obtain mass spectra of these peaks. We supposed that Afc also possessed traces of TMA (Table 2).

Large amounts of unsaturated hydrocarbons could be identified in the lipid extract of *T. angelicae* (Table 1). Analysis of the DMDS derivatives and the results from GC-FTIR revealed that this aphidiid species possessed (Z)-11-alkenes of the chain lengths C_{27}, C_{29}, C_{31}, and C_{33} (peaks 13, 24, 36, and 45). Of these, nonacosene (peak 24) and hentriacontene (peak 36) were the prevalent

alkenes. We assume more unsaturated components on *T. angelicae*, namely, the substances with the peak numbers 22, 33, and 43 (Table 2). Their molecular masses were at *m/e* 402, 430, and 458, respectively. Their fragmentation pattern was typical for polyunsaturated hydrocarbons with the diagnostic ion fragments at *m/e* 55, 67, 81, and 95. Moreover, after DMDS derivatization of the extract, these peaks vanished at their characteristic retention times. Therefore, we propose a triene structure with the chain lengths C_{29} (peak 22), C_{31} (peak 33), and C_{33} (peak 43).

In the case of the parasitoid species *T. angelicae*, some polar lipid components could be detected. Two substances in the lower molecular mass range were indicative of methyl docosanol and methyl tetracosanol (in Figure 1 these peaks are marked by an asterisk). The lipid components in the equivalent chain length of more than C_{33} have not been identified so far, but their mass spectra indicated a nonhydrocarbon nature.

Comparison of the cuticular hydrocarbon profiles of Afc and *L. cardui* revealed that the parasitoid possessed almost all hydrocarbons that could be detected on its host aphid (Table 2). *L. cardui* matched 23 of the total of 25 hydrocarbons on Afc. Eight of these matching compounds belonged to the *n*-alkane fraction, whereas the remaining 15 compounds belonged to the methyl-branched alkanes. Especially in the mass range between C_{23} and C_{30}, the hydrocarbon patterns of host and parasitoid were qualitatively very similar. In the higher molecular weight range of more than 30 carbon atoms, *L. cardui* was characterized by the presence of species-specific hydrocarbons. A quantitative comparison showed less resemblance between Afc and *L. cardui*, with differences in relative abundances mainly expressed in the *n*-alkane and DMA fraction (Table 1). The cuticular hydrocarbon profile of *T. angelicae* corresponded to that of its host aphid in only a dozen compounds. The great majority (eight compounds) belonged to the *n*-alkane fraction, while the remaining four compounds were terminally branched MMA (Table 2).

DISCUSSION

Four observations support the hypothesis that the aphidiid wasp *L. cardui* uses chemical mimicry in order to exploit ant-attended Afc colonies. First, ants behave towards the parasitoid in a similar way as towards the aphids when encountered in the colony; *L. cardui* is either totally ignored or only briefly antennated by the ants. Second, different ant species even belonging to different families show the same behavior towards this parasitoid (Völkl and Mackauer, 1993). Third, *L. cardui* females aggregate in cages and attempt to oviposit in conspecifics when aphid hosts are not present (Starý and Völkl, 1988). Such an oviposition attack does not differ from that towards host aphids. The parasitoid

briefly antennates the host, bends its abdomen ventrally between its legs, and pushes its ovipositor towards the body of the host (C. Liepert, personal observation). This phenomenon shows that *L. cardui* possesses "host" signals on its cuticular surface that elicit species-specific oviposition behavior. Fourth, the acceptance of *L. cardui* by aphid-attending ants is based on cuticular surface lipids (Liepert and Dettner, 1993).

In general, for a chemical mimicry system to work, the signal emitted by the mimic must be indistinguishable for the receiver from the model signal (Dettner and Liepert, 1994). In such cases where there is a complex cuticular hydrocarbon pattern involved, it is not possible to specify which compounds and/or whether their relative abundances are important to fool the signal receiver. In the present example the qualitative and/or quantitative resemblance of the hydrocarbon profiles of *L. cardui* and its host aphid Afc are sufficient for the parasitoid to be considered as "aphid" by the ants. This means that the ants only perceive that portion of the complex cuticular hydrocarbon profile of *L. cardui* that corresponds to the substances on the host aphid, while parasitoid-specific compounds do not play a role in the recognition process. Presumably, components of the mass range between C_{23} and C_{30} might be involved in the communication process, because there is a strong qualitative resemblance between parasitoid and host. The *L. cardui*-specific hydrocarbons are characterized by a molecular weight of more than C_{30}. Similar results were obtained from the beetle *Myrmecaphodius excavaticollis*, an inquiline of *Solenopsis* species (Vander Meer and Wojcik, 1982). The beetle only acquires the host-specific hydrocarbons of lower molecular weight. When living in nests of *S. richteri*, *M. excavaticollis* possesses cuticular substances in the range between C_{22} and C_{28}. As an inquiline of the related species *S. invicta*, the host-specific hydrocarbons on the beetle range from C_{27} to C_{29}. In both cases, the beetle also expresses innate components of higher molecular weight (C_{30}–C_{38}).

In addition to the importance of chain length, additional structural characteristics of hydrocarbons are also of major importance in chemical communication systems in insects. Methyl-branched alkanes definitely represent a substance class with great informative value. In general, methyl alkanes have been demonstrated to be physiologically active in several contexts of insect communication (see Howard, 1993). Moreover, they have been shown to be fairly specific, and changing the methyl position by one or two carbon atoms to either side in the chain markedly reduced kairomonal activity in the host–parasite system of *Helicoverpa zea* and *Microplitis croceipes* (Jones et al., 1971). Branched hydrocarbons also play an important role in communication between ants. Bonavita-Cougourdan et al. (1987), studying the ant species *Camponotus vagus*, demonstrated that methyl-branched alkanes can be involved in nestmate recognition processes. Therefore, we suspect that the presence of host-specific

methyl alkanes on the cuticle of *L. cardui* represents a major cue in its mimicry system. In fact, the parasitoid has 15 methyl-branched alkanes in common with the aphid Afc. Although *n*-alkanes also correspond in the cuticular extracts of aphid and parasitoid, we believe that they are of minor importance in the communication process because they are widely distributed components of the cuticular lipid layer of insects (Lockey, 1988).

Quantitative comparison of the cuticular hydrocarbon profiles revealed less resemblance between Afc and *L. cardui*. However, it is still a matter of conjecture whether such quantitative differences between model and mimic are important in chemical mimicry systems. For example, Howard et al. (1990) also reported differences in relative abundances of cuticular hydrocarbons in the mimicry system of the syrphid *Microdon albicomatus* and its host ant *Myrmica incompleta*. Quantitative differences of cuticular hydrocarbons are believed to play a major role in nestmate recognition processes in ants. Bonavita-Cougourdan et al. (1993), in investigations of the functional subcaste discrimination in the ant species *Camponotus vagus*, postulated that the ants are able to discriminate foragers and brood-tenders by means of quantitative differences in their hydrocarbon profiles.

In the present system we cannot rule out that other cuticular compounds, as well as hydrocarbons, may also be important in the chemical mimicry of the parasitoid *L. cardui*. For example, Franks et al. (1990) pointed out that fatty acids should be included in analyses and thought that they might play a more important role than cuticular hydrocarbons in recognition processes. They showed that the cuckoo ant, *Leptothorax kutteri*, possesses similar cuticular hydrocarbon and fatty acid profiles to workers of its host ant species, *L. acervorum*.

The source of the cuticular hydrocarbons of the aphidiid *L. cardui* is still unknown. Generally, the two possibilities are acquisition or biosynthesis of the substances. For both possibilities there are numerous examples known in the literature (see Dettner and Liepert, 1994). Acquisition by grooming and/or rubbing behavior can be excluded, since we analyzed naive parasitoids that had no prior contact with its host aphids. Moreover, grooming behavior towards host aphids has never been reported for *L. cardui* in the literature and has never been observed by us. The aphidiid parasitoid *Paralipsis eikoae*, however, displays rubbing behavior in order to acquire the odor of *L. niger* ants that attend the parasitoid's host aphid colonies. *P. eikoae* has to succeed in mounting an *L. niger* ant and rubbing its legs on the ant's body in order to be accepted by the ants (Takada and Hashimoto, 1985).

The parasitoid species *T. angelicae* possesses a qualitatively very different hydrocarbon profile compared to its host aphid Afc. The most striking feature of *T. angelicae* is the presence of large amounts of unsaturated hydrocarbons on its cuticle (see Table 1), in particular the homologous series of (*Z*)-11-alkenes

of chain lengths C_{27}, C_{29}, C_{31}, and C_{33}. They represented about 45% of the total hydrocarbon fraction. It is very likely that these compounds induce ant attacks against *T. angelicae*. Preliminary bioassays, in which *T. angelicae*-specific amounts of (*Z*)-11-heptacosene and (*Z*)-11-nonacosene were applied to dead hexane-washed parasitoids and then offered in ant-attended Afc colonies, showed that *L. niger* responded very aggressively towards such test objects (Stegen, Liepert, and Dettner, unpublished data). However, we cannot exclude other chemicals produced in exocrine glands of *T. angelicae* that may be responsible for ant aggression. Exocrine gland secretions from parasitoids can have a modifying effect on the behavior of ants. Völkl et al. (1994) demonstrated that the aphid hyperparasitoid *Alloxysta brevis* uses mandibular secretions to overcome ant aggression when searching for hosts in an aphid colony.

The strategy of using chemical mimicry as an integrating mechanism is very effective in the case of the aphidiid wasp *L. cardui*. It can parasitize almost the entire aphid colony without being molested by attending ants (Völkl, 1992). In general, nonaggressive ant–parasitoid interactions seem to be quite common within the genus *Lysiphlebus*. The specialized *L. hirticornis* and the polyphagous *L. fabarum* show the same behavioral pattern of a cryptic performance as *L. cardui* when foraging in ant-attended host colonies (Starý, 1966; Mackauer and Völkl, 1993; C. Liepert, personal observation), and the polyphagous *L. testaceipes* is at least not attacked by *L. niger* (Völkl and Mackauer, 1993). Therefore, further studies and chemical analyses of host aphids and parasitoids have to be undertaken to clarify whether all *Lysiphlebus* species utilize chemical mimicry and whether the polyphagous species are able to adjust their cuticular hydrocarbon profile to that of the respective host aphid.

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EFFECT OF BIRCH (*Betula pendula*) BARK AND FOOD PROTEIN LEVEL ON ROOT VOLES (*Microtus oeconomus*): I. FOOD CONSUMPTION, GROWTH, AND MORTALITY

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Abstract—The effect of protein and birch bark powder (BBP) content of forage on food consumption and growth of root voles (*Microtus oeconomus*) was studied by feeding young voles with eight different diets for two weeks. Voles ate more when the protein content of forage was moderate (6%) or low (3%), and when BBP was added to the diets, as compared to a high (12%) protein diet without BBP addition. On high and moderate protein diets voles gained weight, whereas on low protein diets they lost weight. Addition of BBP caused reduced growth or weight loss. Consuming a diet with moderate (6%) protein and high BBP (20%) resulted in high mortality (9 of 10 died). These results show that root voles need at least 6% protein for growth and that the effect of birch bark depends on the protein content of the forage.

Key Words—Vole, *Microtus oeconomus*, birch, *Betula pendula*, dietary protein, food consumption, growth, mortality, liver glycogen.

INTRODUCTION

Recent experimental data indicate that plant secondary metabolites and nutrients in the forage influence the consumption of food, growth, and survival of arvicoline (microtine) rodents (Jung and Batzli, 1981; Batzli, 1985; Lindroth et al., 1986). Generalist herbivores usually prefer plant species that simultaneously offer high amounts of nutrients with relatively low levels of secondary com-

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pounds (Bergeron and Jodoin, 1987; Marquis and Batzli, 1989; Bucyanayandi and Bergeron, 1990).

During winter, root voles and other arvicoline voles gnaw the bark of young deciduous and coniferous trees (Tast, 1966; Buchalczyk et al., 1970; Gebczynska, 1970; Korhonen et al., 1983; Spencer, 1984; Batzli and Henttonen, 1990). Gnawing of the bark of birch (*Betula pendula* Roth) is most obvious in winters when high numbers of voles are present (Hansson, 1986, 1989), probably because more palatable food items are scarce or unavailable (Tast, 1966; Korhonen et al., 1983; Bergeron, 1984). Birch bark is not rich in protein (Palo et al., 1985; Harju and Tahvanainen, 1994), but it contains high amounts of plant secondary compounds, such as phenolics and terpenoids (Tahvanainen et al., 1991). Feeding on forage low in protein and rich in secondary chemicals was recently shown to affect field voles (*Microtus agrestis* L.) adversely (Harju and Tahvanainen, 1994).

Here I report an experiment on the interaction of dietary protein and birch bark powder (BBP) using young root voles as experimental animals. BBP was used as a source of secondary compounds in the experimental diet. Voles were fed for two weeks with eight different diets containing three levels of protein and BBP. The hypotheses tested were: (1) low dietary protein in interaction with birch bark powder reduces growth of animals and increases intake of forage (see Harju and Tahvanainen, 1994), and (2) high dietary protein compensates for the adverse effects of birch bark powder.

METHODS AND MATERIALS

The experimental diets were prepared by adding dried BBP in food pellets. For the preparation of BBP, twigs of the current year's growth of young, winter-dormant silver birch (*Betula pendula* Roth) (up to 2.5 m tall) were collected in November 1988 near Joensuu, in eastern Finland. The resinous twigs (< 10 mm in diameter) were peeled and the bark was dried in a ventilated oven at 48°C for 20 hr. The dried bark was ground and the powder stored frozen (−20°C) until used.

The crude protein concentrations of the food pellets used were 3%, 6%, and 12% (manufacturer: FinnEwos). Eight diets were prepared (Table 1) by mixing ground pellets with water, a small amount of potato flour, and different amounts of birch bark powder (0%, 5% and 20%, w/w). Pellets weighing 20–30 g were dried in a ventilated oven at 48°C for 20 hr.

The voles used were first- to third-generation offspring from a stock of root voles (*Microtus oeconomus* Pallas) maintained at the University of Joensuu. The original population was brought from Pallasjärvi, northern Finland. The parent population received ad libitum commercial pellets for laboratory animals (crude

TABLE 1. PROTEIN AND BBP CONTENT OF DIETS AND MORTALITY OF EXPERIMENTAL ROOT VOLES WITHIN EACH TREATMENT

Treatment	Protein content (%)	BBP content (%, w/w)	Mortality (individuals)
3/0	3	0	0/10
3/5	3	5	0/10
6/0	6	0	1/10
6/5	6	5	4/10
6/20	6	20	9/10
12/0	12	0	1/10
12/5	12	5	0/10
12/20	12	20	4/10

protein content about 20%), water, and slices of potatoes and carrots as fresh food.

Young voles were weaned at the age of 18 days and kept as littermate groups. They received water and commercial pellets ad libitum until the beginning of the experiment. At about 5 weeks of age (range 4–8 weeks) individuals were randomly divided among the experimental groups (Table 1) and started on their respective diets. Each group consisted of 10 individuals. The sex of the voles was not recorded.

During the experiments the voles were housed individually in plastic cages (22 × 27 × 10 cm) with hardwood chip bedding. Fresh water was given every day in a plastic cup inside the cage. The temperature was kept at 18–19°C, and the light cycle was 6L:18D. Both the voles and their food were weighed daily. The experiment lasted for 14 days. At the end of the experiment the voles were anesthesized with ether and killed by dislocating the neck.

The glycogen content of liver tissue, used as an approximate index of nutritional status, was measured by the method described by Lo et al. (1970). A piece of liver tissue weighing about 100 mg was hydrolyzed with 1 ml of 30% KOH (saturated with Na_2SO_4) for 20–30 min in a boiling water bath until a homogenous solution was obtained. Samples were cooled in ice. Glycogen was precipitated from the alkaline digestae by adding 1.2 ml of 95% ethanol. Samples were kept on ice for a further 30 min. After centrifugation (3000 rpm, 25 min) the supernatant was removed and the remaining glycogen precipitate was dissolved in 10 ml of distilled water. Then 0.05 ml of this solution was brought to a sample volume of 1 ml by adding distilled water, and 1 ml of 5% phenol and 5 ml of 96–98% H_2SO_4 were added to the solution. After standing for 10 min, the samples were shaken and placed for 10–20 min in a water bath

(25–30°C). The absorbance was read at 490 nm with Hitachi U-2000 spectrophotometer. All samples were analyzed in duplicate. Results were read from the glycogen standard curve (glycogen stock solution 5 mg/ml distilled water).

Statistical tests were performed with SAS (Freund et al., 1986). The effect of age, initial body mass, and diet on the survival of voles was tested with the LOGISTIC procedure. The food consumption and growth data were tested using the GLM repeated measures procedure for analysis of variance, using type IV sums of squares (Freund et al., 1986), and the liver glycogen data were tested using two-way ANOVA. The two variables were dietary protein content and birch bark powder concentration. Individuals that did not survive through the experimental period were excluded from the analyses.

RESULTS

The more BBP the forage contained, the less likely the root voles were to survive ($\chi^2 = 14.0$, $P < 0.001$) through the experiment (Table 1). Protein as such did not affect survival ($\chi^2 = 0.27$, $P = 0.6067$). The age ($\chi^2 = 1.39$, $P = 0.2393$) or the initial body mass ($\chi^2 = 0.004$, $P = 0.9528$) of nonsurviving voles did not differ significantly from those of the surviving voles.

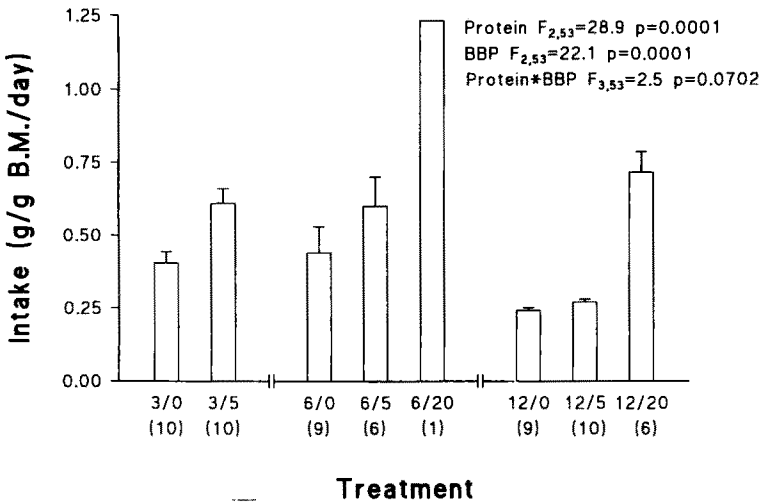

FIG. 1. Mean daily consumption of food by surviving root voles ($X \pm$ SE). Treatments as in Table 1. Number of individuals in parentheses.

Consumption of food was affected significantly (Figure 1) by both the protein level ($F_{2,53} = 28.9$, $P = <0.001$) and BBP concentration of the forage ($F_{2,53} = 22.1$, $P < 0.001$). There was a nearly significant interaction between dietary protein and BBP ($F_{3,53} = 2.5$, $P = 0.0702$). The consumption was lowest on the high (12%) protein diet without BBP. On all protein levels, addition of BBP into the diet increased consumption. The consumption was highest on diets containing 20% BBP. Individual voles within high BBP treatments (groups 6/20 and 12/20), however, responded to the diets in two different ways. The first group had very low intake (average daily intake 0–6 g) and a short survival time (1–2 days, $N = 10$). The other group of animals had very high intake (average daily intake 12–26 g) and longer survival time (4–14 days, $N = 10$) with seven individuals surviving through the experiment.

Growth of voles was affected by both the protein and BBP content of the diet (protein $F_{2,53} = 17.8$, $P < 0.001$; BBP $F_{2,53} = 3.1$, $P = 0.0531$). There was no significant interaction between dietary protein and BBP ($F_{3,53} = 1.1$, $P = 0.3135$). Growth was best on high (12%) protein diets, and a decrease in protein content caused slower growth or loss of weight (Figure 2). Despite

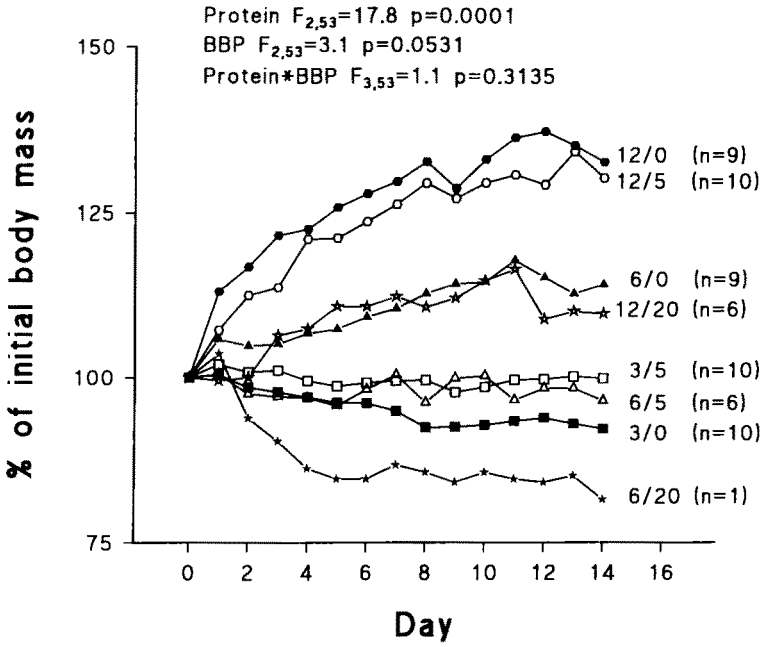

FIG. 2. Mean body mass of surviving root voles, shown as percent of initial body mass, in different treatments. Treatments as in Table 1.

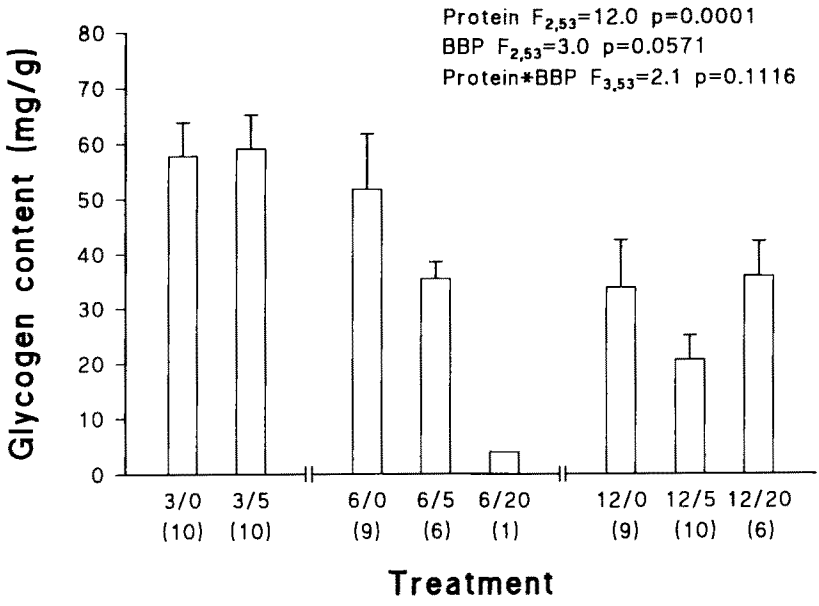

FIG. 3. The mean ($X \pm$ SE) glycogen content of liver tissue of surviving root voles. Treatments as in Table 1. Number of individuals in parentheses.

increased consumption, the voles gained less or even lost weight when BBP was added to the forage, with the exception of groups 3/0 and 3/5. On the diet containing 6% protein and 20% BBP, only one individual survived, losing about 20% of its weight in two weeks.

The glycogen content of the livers (Figure 3) was low on high protein diets as compared with low protein diets ($F_{2,53} = 12.0$, $P < 0.001$). The increase in BBP concentration tended to result in lower glycogen content ($F_{2,53} = 3.0$, $P = 0.0568$) as compared to diets without BBP addition. There was no significant interaction between dietary protein and BBP ($F_{3,53} = 2.1$, $P = 0.1116$).

DISCUSSION

Feeding on diets containing 20% BBP resulted in heavy mortality of experimental voles (Table 1). Poor survival of voles when fed forage containing secondary compounds was reported also by Lindroth and Batzli (1984) and Jung and Batzli (1981). In this experiment the voles that did not survive usually refused to eat or ate only very little, and died within one to two days (six voles

in group 6/20 and four in group 12/20). This suggests that the death of animals was caused by starvation, not by the toxicity of the added BBP. However, in a few cases (three voles in group 6/20) voles ate high amounts and yet died. These voles were probably unable to handle the secondary compounds received from BBP in the forage, and the mortality here was apparently due to the toxic effects of BBP. According to Lindroth and Batzli (1984), meadow voles need at least 8% protein in forage to detoxify phenolic-rich diets. Low protein diets were also reported by Dauterman (1980) and Sipes and Gandolfi (1986) to decrease the ability of animals to metabolize plant toxins.

In the present experiment there was a clear increase in consumption of food when dietary protein content decreased from 12% to 6%. There was no further increase when protein content decreased from 6% to 3%. On the other hand, an increase in consumption was seen also when concentration of BBP was increased. Increased consumption of low-quality food, as seen in this study, was probably due to an effort to meet the nutritional requirements (Batzli and Cole, 1979; Batzli, 1985) in the absence of alternative food. However, whatever the demand for nutrients, food cannot be ingested faster than the gut can process it (Batzli, 1985). At the highest intake rates (groups 6/20 and 12/20), the voles consumed about their own weight of forage per day, which might be the limit set by the gut (Batzli, 1985).

The increase in dietary concentration of BBP, while promoting consumption, significantly decreased the growth of voles. This and low liver glycogen content indicate an increased metabolic cost in processing these diets (Thomas et al., 1988). On low protein diets (with or without BBP) the voles apparently did not gain enough protein for growth, hence they stored glycogen (=energy). On high protein diets, on the other hand, the glycogen in liver was probably used as an energy source for growth. The lower glycogen content when BBP was added to the diets was probably due to the increased need for energy to metabolize the secondary compounds of BBP. The compensatory effect of high dietary protein content has previously been reported for rats also (Glick and Joslyn, 1970). According to Jean and Bergeron (1986), voles seem to tolerate secondary compounds of commonly eaten plants but seem to be adversely affected when nutrients such as proteins are low in the forage.

In conclusion, these results show that the bark of birch twigs is a low quality forage for voles (see also Harju and Tahvanainen, 1994). In general, voles are well adapted to utilizing food high in fiber and low in protein (Batzli, 1985; Hume et al., 1993). However, when secondary compounds are present in high quantity, the performance of voles seems to be dependent on the concentration of dietary protein. Low dietary protein in interaction with BBP reduces the growth of the animals and increases their consumption of food. The results presented here show that a high dietary protein level can compensate for the harmful effects of birch bark powder.

The wintering success of root voles seems to depend on their ability to maintain their body mass (Tast, 1972, 1984), which has been shown to correlate with the quality/quantity of winter forage (Tast, 1984). According to Tast (1972) and Cole and Batzli (1979), the quality of available food can exert a strong influence on the density of herbivores. This leads to the suggestion that utilizing bark of birch or other trees as winter forage may cause serious problems to root voles and may affect the population dynamics during high vole densities.

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EFFECT OF BIRCH (*Betula pendula*) BARK AND FOOD PROTEIN LEVEL ON ROOT VOLES (*Microtus oeconomus*): II. DETOXIFICATION CAPACITY

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Abstract—The effect of protein and birch bark powder (BBP) content of forage on detoxification capacity of root voles (*Microtus oeconomus*) was studied. Young voles were fed with eight different diets for two weeks. Individuals on low (3%) protein diets had significantly lighter livers and kidneys than those on moderate (6%) or high (12%) protein diets. Birch bark powder addition did not have significant effect on organ weight. Detoxification was significantly induced, apparently due to secondary compounds in BBP. The activity of ethoxyresorufin-*O*-dealkylase (EROD) was high when protein content or BBP concentration in forage was high as compared to low protein diets or diets containing no BBP. Glucuronidation, on the other hand, was not induced by BBP. High BBP content caused serious physiological stress to the voles. The only individuals surviving were those capable of sufficiently allocating energy and protein to detoxification.

Key Words—Voles, *Microtus oeconomus*, birch, *Betula pendula*, dietary protein, detoxification capacity, EROD, glucuronidation.

INTRODUCTION

Mammalian herbivores utilize several physiological mechanisms to overcome the adverse effects of plant secondary compounds (Freeland and Janzen, 1974). One of these mechanisms is a two-phase detoxification system located mainly in the liver (Bidlack and Smith, 1984; Sipes and Gandolfi, 1986). Addition of functional groups (phase I) to the compound provides reactive sites for the conjugative (phase II) reactions (Freeland and Janzen, 1974; Sipes and Gandolfi,

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1986; Siess et al., 1988). In phase I the relatively nonspecific cytochrome P-450-containing monooxygenases (Sipes and Gandolfi, 1986), such as ethoxyresorufin-*O*-dealkylase (EROD), carry out oxidation, hydroxylation, and reduction. In phase II different transferases conjugate compounds with, e.g., glucuronic acid or inorganic sulfate (Caldwell, 1986). These metabolic alterations produce more hydrophilic, more readily excreted, and generally less toxic compounds as compared with the parent compound(s) (Freeland and Janzen, 1974; Caldwell, 1986; Sipes and Gandolfi, 1986).

The nutritional value of the diet seems to influence the ability of herbivores to detoxify plant secondary compounds (Schwass and Finley, 1985; Sipes and Gandolfi, 1986). Nutritional deficiencies (e.g., lack of protein or minerals) may reduce the activity of oxidative and conjugative enzymes (Schwass and Finley, 1985; Bidlack and Smith, 1984; Bidlack et al., 1986; Sipes and Gandolfi, 1986; but see Sell and Rogler, 1983). Ingestion of plant secondary compounds, on the other hand, can induce these enzymes (Bidlack and Smith, 1984; Chadha and Madyastha, 1984; Sipes and Gandolfi, 1986; Alldrick et al., 1988; Siess et al., 1988; Harju and Tahvanainen, 1994), which allow herbivores to alleviate the harmful effects of those compounds. Detoxification is energetically expensive, and there is a limit to the extent the enzymes can be induced (Freeland and Janzen, 1974; Harborne, 1991). This limit should be dependent on the nutritional quality of the diet.

As a secondary source of food, arvicoline (microtine) voles gnaw the bark of young deciduous trees, such as birch (*Betula* spp.) (Buchalczyk et al., 1970; Korhonen et al., 1983; Batzli and Henttonen, 1990). The bark of birch and other woody species is gnawed especially in winters with high vole densities (Hansson, 1986), probably because more palatable food is scarce (Korhonen et al., 1983; Bergeron, 1984). Consuming birch bark causes voles serious stress and leads to poor growth and survival of animals, although intake of food increases (Harju and Tahvanainen, 1994; Harju, 1996).

Birch bark is poor in protein (Palo et al., 1985) but rich in secondary compounds such as terpenoids and phenolics (Tahvanainen et al., 1991). Both phenolics and terpenoids are shown to be metabolized (phase I) by cytochrome P-450 monooxygenases (Scheline, 1978; Watabe et al., 1980; Gordon et al., 1987; McLean et al., 1993) and conjugated (phase II) with glucuronic acid (Scheline, 1978; McLean et al., 1993). In the present experiment, birch bark powder (BBP) was used as a source of secondary compounds in the diet. This paper reports an experiment on the effect of BBP ingestion and protein content of forage on the detoxification capacity of young root voles (*Microtus oeconomus* Pallas). The hypotheses tested were: (1) secondary compounds in BBP should induce detoxification (Harju and Tahvanainen, 1994), and (2) high dietary protein should improve detoxification.

METHODS AND MATERIALS

Root voles, about 5 weeks old, were fed for two weeks with eight different diets (Table 1). During the feeding voles were housed individually in plastic cages (22 × 27 × 10 cm). Details of the preparation of the diets and the feeding trial are reported in Harju (1996). At the end of the experiment voles were killed by dislocating the neck. Liver and kidneys were removed and weighed, and a piece of liver was stored in liquid nitrogen until used. The substrates and other biochemicals for the chemical assays were manufactured by Sigma Chemical Company.

The microsomal fraction of hepatic tissue was prepared as in Burke and Mayer (1974), with slight modifications. The tissue was homogenized in 0.25 M sucrose (1 g:4 ml). The homogenate was centrifuged at 4°C and 10,000*g* for 20 min, and the resulting supernatant was centrifuged at 4°C and 105,000*g* for 60 min. The microsomal pellets were resuspended in 0.25 M sucrose, 0.05 M Tris buffer, pH 7.4 (HCl), 1 ml corresponding to 1 g of original tissue sample. The microsomal suspensions were stored at −80°C until used. The protein content of microsomes was measured by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Both enzyme assays were performed at 37°C. The activity of EROD was measured from the microsomes as described in Lubet et al. (1985). The reaction mixture contained 25 μl of microsomal suspension, 1875 μl of Tris HCl buffer (0.1 M, pH 7.8), and 100 μl of 10 μM ethoxyresorufin. The activity was measured at an excitation wavelength of 530 nm and an emission wavelength of 585 nm with a Shimadzu RF-5000 spectrofluorophotometer. The reaction was started by adding 20 μl of 5 mM NADPH. After 3 min, 20 μl of 0.1 mM

TABLE 1. PROTEIN AND BBP CONTENT OF DIETS AND MORTALITY OF EXPERIMENTAL ROOT VOLES WITHIN EACH TREATMENT

Treatment	Protein content (%)	BBP content (%, w/w)	Mortality (individuals)
3/0	3	0	0/10
3/5	3	5	0/10
6/0	6	0	1/10
6/5	6	5	4/10
6/20	6	20	9/10
12/0	12	0	1/10
12/5	12	5	0/10
12/20	12	20	4/10

resorufin was added as an internal standard. The activity was found to be linear over the time period used for the assay. Duplicate determinations of activity were carried out on the same liver extract. Enzyme activity was expressed as nanomoles of substrate hydrolyzed per individual per minute.

With *p*-nitrophenol (*p*-NP) as an aglycone and uridine 5′-diphosphoglucuronic acid (UDPGA) as the glucuronyl donor, UDP-glucuronosyltransferase (UDP-GT) activity of microsomes was measured as described in Hänninen (1968). The reaction mixture of 0.1 ml potassium phosphate buffer (0.5 M, pH 7.0) containing 0.35 mM *p*-NP and 6.89 mM UDPGA, and 0.2 ml of sucrose-diluted microsomes (25 μl of original microsome suspension), was incubated for 20 min. The reaction was stopped by adding 900 μl of cold 3% tricholoroacetic acid. The mixture was centrifuged and the supernatant solution added to 100 μl of 5 M NaOH. The absorbancy was measured immediately at 400 nm with a Hitachi U-2000 spectrophotometer. The activity was found to be linear over the time period used for the assay. Duplicate determinations of activity were carried out on the same liver extract. Enzyme activity was expressed as nanomoles of *p*-NP glucuronylated per individual per minute.

Results were tested with the SAS GLM-procedure for two-way analysis of variance (Freund et al., 1986). The variables were dietary protein content and birch bark powder concentration. The initial body mass of voles was used as a covariate in analyses of liver and kidney mass (Shirley, 1977).

RESULTS

Individuals on low (3%) protein diets had lighter livers and kidneys than voles on other diets (Figure 1, Table 2). Dietary BBP did not have a significant effect on either liver or kidney mass. There was no significant interaction between protein and BBP, despite the different trends seen on moderate (6%) and high (12%) protein diets.

The protein content of hepatic microsomes (Figure 2) was significantly higher on high protein diets than on low protein diets (Table 2). BBP did not have a significant effect on microsomal protein content, and there was no interaction between protein and BBP content of the diet (Table 2).

The capacity of EROD was higher on high protein diets than on low protein diets, and dietary BBP caused a significant increase of capacity (Figure 3, Table 2). The only surviving individual in group 6/20 had the highest EROD capacity. There was also a significant interaction between the protein and BBP content in the diet. Capacity of UDP-GT, on the other hand, was affected only by dietary protein (Figure 4, Table 2). Dietary BBP did not have a significant effect on UDP-GT capacity.

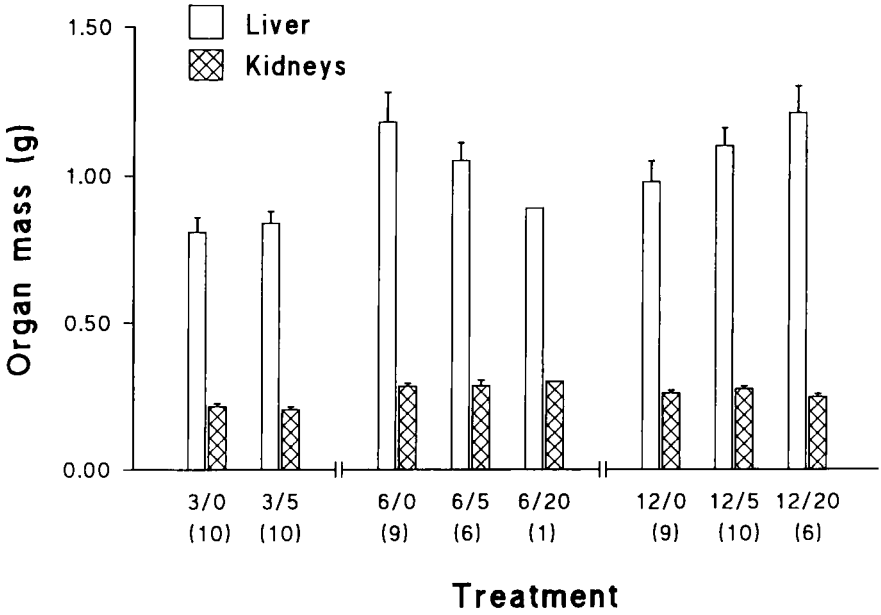

FIG. 1. Mean mass ($X \pm$ SE) of liver and kidneys of surviving root voles. Treatments as in Table 1. Number of individuals in parentheses.

TABLE 2. RESULTS OF TWO-WAY ANOVA FOR EFFECTS OF PROTEIN AND BBP ON MASS OF LIVER AND KIDNEYS, MICROSOMAL PROTEIN, AND MICROSOMAL EROD AND UDP-GT CAPACITY OF ROOT VOLES

	Effect of protein	Effect of BBP	Interaction
Liver mass	$F = 5.60$	$F = 0.14$	$F = 2.42$
	$P = 0.0063$	NS	NS
Kidney mass	$F = 30.92$	$F = 0.20$	$F = 0.77$
	$P = 0.0001$	NS	NS
Microsomal protein	$F = 10.16$	$F = 1.83$	$F = 1.28$
	$P = 0.0002$	NS	NS
EROD capacity	$F = 20.60$	$F = 22.96$	$F = 9.86$
	$P = 0.0001$	$P = 0.0001$	$P = 0.0001$
UDP-GT capacity	$F = 4.61$	$F = 0.68$	$F = 0.17$
	$P = 0.0142$	NS	NS

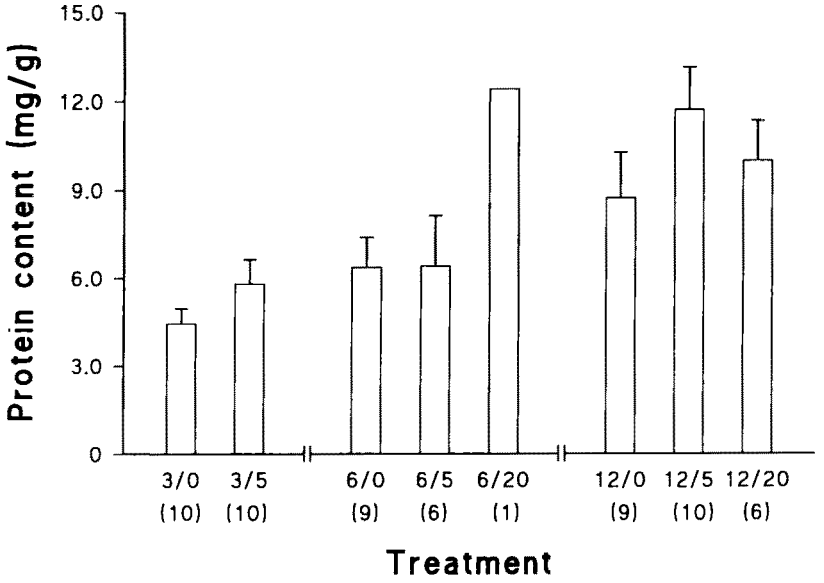

FIG. 2. Mean microsomal protein content (milligrams of protein per gram of liver tissue, $X \pm$ SE) of surviving root voles in different treatments (Table 1). Number of individuals in parentheses.

DISCUSSION

Significant changes in liver or kidney mass due to BBP in the forage were not seen in this study. Recent reports on the effects of plant secondary compounds indicate that increasing the concentration of BBP in the diet might have been expected to result in an enlarged liver to detoxify the secondary compounds and enlarged kidneys to process the resulting wastes (Jung and Batzli, 1981; Bergeron and Jodoin, 1982, 1984; Konarzewski and Diamond, 1994). In this study there might have been a hypertrophy of metabolically active tissue but a concomitant depletion of glycogen (Harju, 1996) and/or fat reserves may have resulted in apparently unchanged liver/kidney mass (Konarzewski and Diamond, 1994).

The lower microsomal protein content on low protein diets suggests that there was a protein deficiency in the liver that limited the capacity of the detoxification system on these diets (Figure 3). Inadequate protein in the diet has been reported to decrease significantly hepatic phase I detoxification activity in rats (Schwass and Finley, 1985; Sipes and Gandolfi, 1986). There was an increase in UDP-GT capacity when dietary protein content increased, suggesting

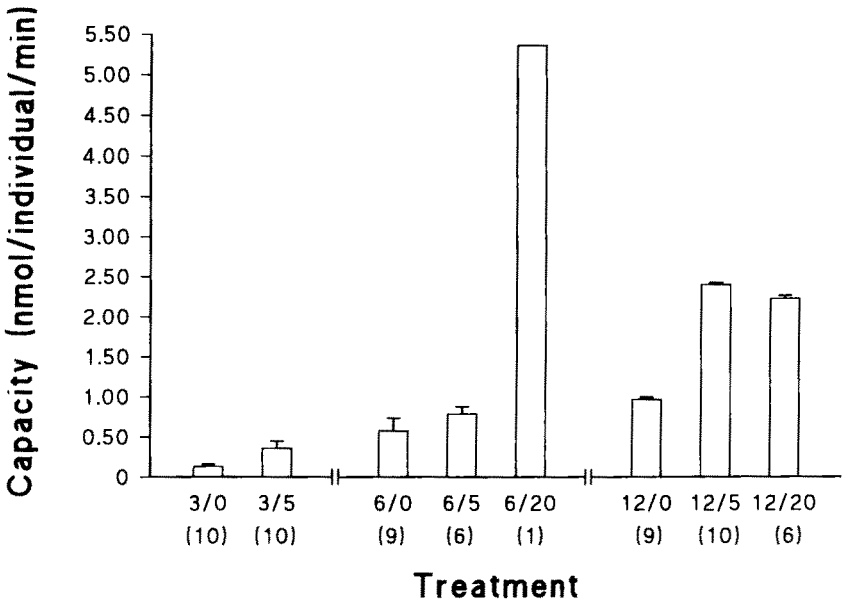

FIG. 3. Mean capacity of EROD (nanomoles of substrate hydrolyzed per individual per minute) of surviving root voles in different treatments (Table 1). Values expressed as mean ± SE. Number of individuals in parentheses.

that UDP-GT activity also would have been protein limited. Dietary BBP, on the other hand, had no significant effect. This is inconsistent with the results showing that glucuronidation activity may increase when availability of dietary protein decreases (Sell and Rogler, 1983) or when animals are exposed to secondary compounds (Siess et al., 1988). The dose of secondary compounds may have not been large enough in this study to induce the phase II reactions. Another alternative is that the compounds produced in phase I were conjugated with another endogenous substrate. Low doses of phenolics, for example, have been shown to conjugate with inorganic sulfate preferentially (Sipes and Gandolfi, 1986).

The detoxification enzymes are induced on the first encounter with xenobiotics such as plant secondary compounds (Harborne, 1991), increasing the capacity of the animal to handle these substances. In this study, a high concentration of BBP in the diet induced phase I detoxification capacity significantly, thus giving partial support to the first hypothesis. Low dietary protein seemed to limit the detoxification capacity, thus supporting the second hypothesis. Individuals capable of inducing detoxification enzymes seem to be less susceptible

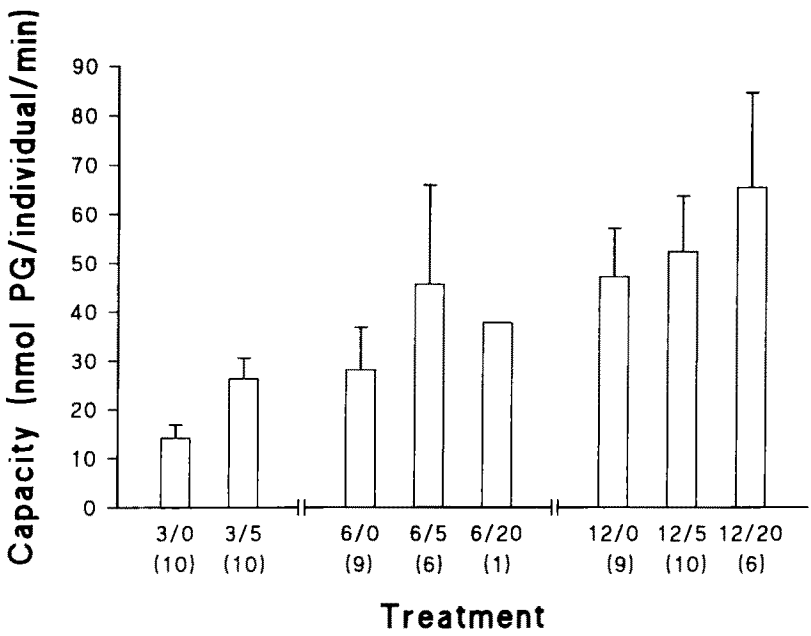

FIG. 4. Mean capacity of UDP-GT (nanomoles of substrate glucuronylated per individual per minute) of surviving root voles in different treatments (Table 1). Values expressed as mean ± SE. Number of individuals in parentheses.

to dietary poisoning (Brattsten et al., 1977). High dietary protein concentration together with induced detoxification enhanced the ability of voles to survive the exposure to BBP containing secondary compounds (see Harju, 1996).

Voles can compensate for a reduction in dietary protein by increasing their intake of food (Harju, 1996). This compensatory feeding causes an increase in consumed BBP (and secondary compounds therein), and thus increases physiological stress. At a certain ratio of protein/BBP concentration the detoxification capacity and physiological tolerance of voles seems to collapse, resulting in high mortality (Harju, 1996). This may happen in times of high population densities when woody plant material containing a low concentration of protein and high amounts of secondary compounds constitutes a high proportion of the diet of voles.

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IDENTIFICATION OF PRESUMED PHEROMONE BLEND FROM AUSTRALASIAN PREDACEOUS BUG, *Oechalia schellenbergii* (HETEROPTERA: PENTATOMIDAE)

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Abstract—*Oechalia schellenbergii* is one of the most common predatory insects in Australia and the islands of the South Pacific. Adult males of this predaceous "true bug" collected during March near Gatton, Queensland, Australia, had a pair of enlarged exocrine glands opening underneath their wings that presumably produce an attractant pheromone. The two major components of the secretion are 3-methylenehexyl acetate and 9-hydroxygeranyl diacetate [2,6-dimethyl-2(*E*),6(*E*)-octadien-1,8-diol diacetate].

Key Words—Predator, Hemiptera, Asopinae, stink bug, geraniol, diacetate.

INTRODUCTION

Augmentation of predaceous pentatomids (Pentatomidae: Asopinae) is a promising biological control tactic for vegetable crop pests (Biever and Chauvin,

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1992a,b; Hough-Goldstein and Keil, 1991), but large numbers of predators must be available to growers at reasonable cost for this approach to be a viable strategy (Hough-Goldstein and Whalen, 1993). Stink bugs that prey on many different insect species are the most common, wide-ranging asopines (McPherson, 1982). Moreover, these abundant predators are often exceptionally reliant on pheromones to congregate (Aldrich, 1988, 1995), perhaps because volatiles associated with specific prey are too varied to be reliable aggregating cues for generalist predators. The availability of synthetic pheromones for general predators offers a means of managing wild populations of these beneficial insects for improved biological pest control.

In Australia and New Zealand, *Oechalia schellenbergii* (Guérin-Méneville) is an important predator of several larval Lepidoptera (Awan, 1988) and Coleoptera (Edwards and Suckling, 1980). One of us (J. R. A.), while on sabbatical in Australia, had the opportunity to initiate a semiochemical investigation of *O. schellenbergii*. Here we report the identification of the suspected attractant pheromone blend of this general predator, including several compounds heretofore unknown as natural products.

METHODS AND MATERIALS

Laboratory Analysis. Some *Oechalia* adults were reared on locally collected caterpillars from eggs of a female collected in November 1990 at the Maroochy Horticultural Research Station, Nambour, Queensland. Other adult predators used for the study were collected in alfalfa fields near Gatton, Queensland, on March 19, 1991, and April 26, 1994, and were dissected within two days of being caught.

Dorsal abdominal glands were excised from CO_2-anesthetized bugs submerged in tap water, and macerated with forceps in ca. 150 μl of CH_2Cl_2 (glands from 6 and 7 males were pooled per sample). After standing at least overnight, the solution was dried with Na_2SO_4, and transferred to a clean vial prior to analysis. Most samples were analyzed on DB-5 and DB-1 (J&W Scientific, Folsom, California) columns (0.25 μm film, 30 m $\times$ 0.25 mm ID) in a Varian 3500 gas chromatograph (GC) with helium as carrier (50 cm/sec), a temperature program from 50°C for 2 min to 235°C at 15°/min, with a flame ionization detector, and recording data using the Varian GC Star Workstation (Sunnyvale, California) software on a Gateway 2000 386/25 computer (North Sioux City, South Dakota). The GC trace (Figure 1) excludes the solvent peak and normalizes peaks to the most abundant natural product. Electron impact-mass spectra (EI-MS) were obtained using a Hewlett Packard 5971 GC-MS instrument at 70 eV, with a HP-5 (Kennett Square, Pennsylvania) column (0.11 μm film; 25 m $\times$ 0.2 mm ID), programmed from 50°C for 2 min to 250°C at 15°/min.

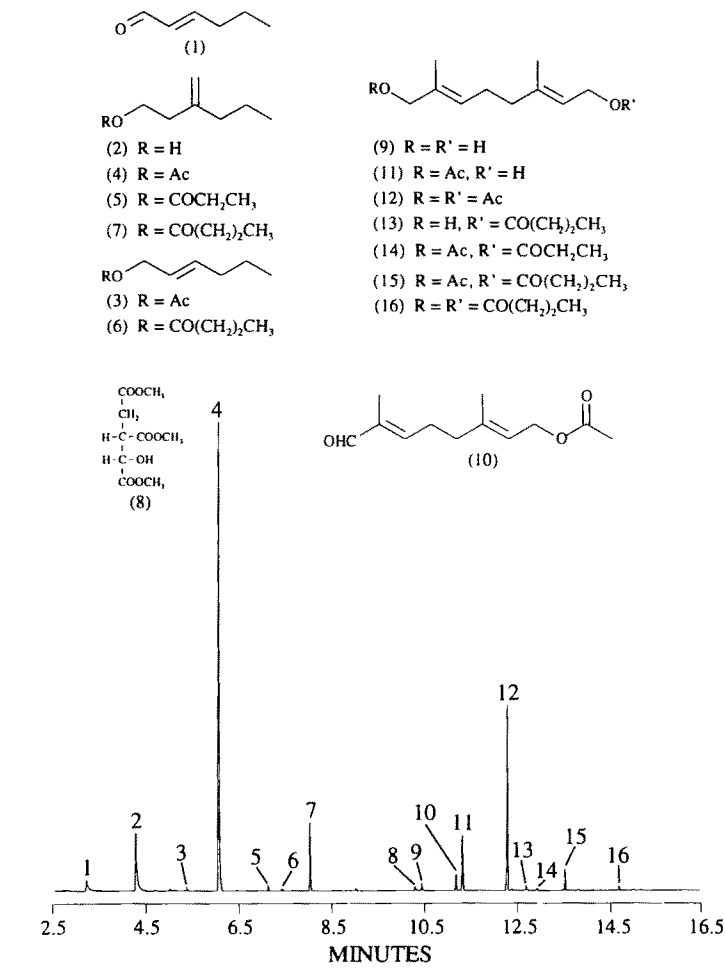

FIG. 1. Gas chromatogram of male *Oechalia schellenbergii* (Heteroptera: Pentatomidae) dorsal abdominal gland secretion.

Chemical ionization-mass spectra (CI-MS) were collected using a Finnigan 4510 GC-MS with an INCOS Data System, a 30-m DB-1 column programmed from 60°C for 2 min to 250°C at 5°/min, and either NH_3 or ND_3 as reagent gas. ^1H NMR spectra were recorded in $CDCl_3$ at 300 MHz on a General Electric QE-300 instrument with TMS as an internal standard. Assignments were made with the COSY technique.

Commercial and Synthetic Standards. All compounds reported in Figure 1, except for **5** and **14,** were verified by mass spectra and by coelution on 30-m DB-5 and/or 60-m DB-1 columns. 3-Methyl-1-hexanol was purchased from Narchem Corp. (Chicago, Illinois), and geranyl acetate was purchased from Givaudan Corp. (Clifton, New Jersey); other standards and reagents were obtained from either Bedoukian Research Inc. (Danbury, Connecticut) or Aldrich Chemical Company (Milwaukee, Wisconsin). The general scheme for preparation of the terpenoid synthetic standards is shown in Figure 2. Sodium borohydride reductions were conducted by adding the aldehyde to a suspension of $NaBH_4$ in absolute ethanol (approximately 0.5 mg $NaBH_4$/mg of aldehyde), stirring at room temperature 1 hr, carefully adding a few drops of acetic acid, and partitioning between pentane and water. Esterifications of primary alcohols were performed by allowing the alcohol to react with an acid chloride or anhydride at room temperature in the presence of pyridine (1–2 hr followed by partitioning into pentane). Saponifications were conducted by KOH in aqueous methanol at room temperature followed by dilution with water and partitioning. Selenium dioxide oxidations were performed as described by Hirata et al. (1981). The trimethylester of isocitric acid was prepared via diazomethane esterification of isocitric acid.

3-Methylenehexan-1-ol (2) and 3-Methylenehexyl Acetate (4). Standards of **2** and **4** were prepared by repeating an ene reaction between 2-methyl-1-pentene

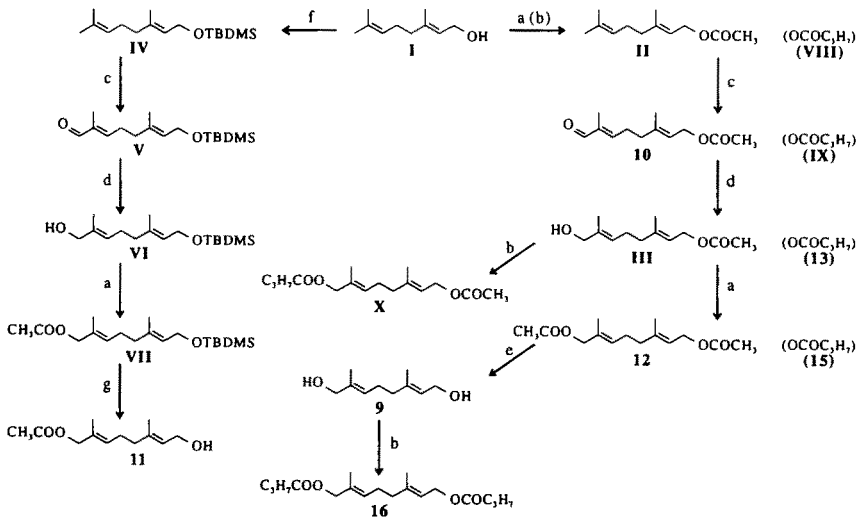

FIG. 2. Scheme for preparation of terpenoid synthetic standards.

and trioxane (Snider et al., 1982). A hexanes solution of dimethylaluminum chloride was used instead of the dichloromethane solution originally described. The three unsaturated alcohols constituted 97% of the products detected by GC (93% yield), and the major (55%), earliest eluting (DB-1 column) isomer was identical by GC coelution and mass spectrometry to the *Oechalia* C_7 alcohol (**2**); EI-MS [*m/z* (%)]: 96 (M − H_2O, 15%), 81 (100), 71 (12), 69 (25), 68 (20), 67 (27), 56 (40), 55 (53), 54 (16), 53 (13), 51 (6), 43 (14), 42 (15), 41 (55), 39 (30); CI-MS (NH_3) [*m/z* (%)]: 166 [M + $(NH_3)_3H$, 7%], 149 [M + $(NH_3)_2H$, 100], 132 (M + NH_4, 45); CI-MS (ND_3) [*m/z* (%)]: 177 [M + $(ND_3)_3D$ − H + D, 18], 157 [M + $(ND_3)_2D$ − H + D, 100], 137 (M + ND_4 − H + D, 25). Acetylation of a portion of the sample gave a mixture of acetates, of which the first eluting by GC (DB-1 column) was identical by retention time and mass spectrometry to **4** (3-methylenehexyl acetate); EI-MS [*m/z* (%)]: 96 (17%), 82 (4), 81 (57), 79 (4), 73 (3), 69 (6), 68 (60), 67 (21), 56 (3), 55 (10), 54 (9), 53 (6), 43 (100), 41 (14), 39 (12); CI-MS (NH_3) [*m/z* (%)]: 330 (2M + NH_4, 5%), 208 [M + $(NH_3)_3H$, 8], 191 [M + $(NH_3)_2H$, 100], 174 (M + NH_4, 57), 157 (M + H, 3). Flash chromatography of the mixture of acetates on silica gel followed by silver nitrate-impregnated silica gel (20% by weight $AgNO_3$) chromatography, eluting successively with 2, 4, and 6% ethyl acetate in cyclohexane, gave about 10 mg of pure **4** (the latest eluting of the three isomers) for ^{1}H NMR spectroscopy; ^{1}H NMR ($CDCl_3$): δ 0.91, t, J = 7.5 Hz, Me6; 1.46, m, two H5; 2.01, t, J = 7.5 Hz, two H4 (partially obscured by Ac Me); 2.04, s, Ac Me; 2.33, t, J = 6.9 Hz, two H2; 4.17, t, J = 6.9 Hz, two H1; 4.49, app. d, J = 10.2 Hz, two H7.

3-Methylenehexyl Propionate (5). EI-MS [*m/z* (%)]: 96 (23%), 82 (7), 81 (66), 79 (3), 69 (8), 68 (93), 67 (26), 57 (100), 55 (19), 54 (13), 53 (9), 43 (13), 41 (18), 39 (9).

3-Methylenehexyl Butyrate (7). Synthetic **7** was derived from **2** by treatment with butyryl chloride; EI-MS [*m/z* (%)]: 96 (24), 82 (5), 81 (73), 79 (3), 72 (3), 71 (60), 69 (8), 68 (100), 67 (20), 55 (20), 54 (9), 53 (5), 43 (49), 42 (6), 41 (27), 39 (13); CI-MS (NH_3) [*m/z* (%)]: 386 (2M + NH_4, 2%), 236 [M + $(NH_3)_3H$, 6], 219 [M + $(NH_3)_2H$, 100], 202 (M + NH_4, 58), 185 (M + H, 4).

2,6-Dimethyl-2(E),6(E)-octadien-1,8-diol (9-Hydroxygeraniol) (9), 8-Oxo-3,7-dimethyl-2(E),6(E)-octadienyl Acetate (10), 2,6-Dimethyl-2(E),6(E)-octadien-1,8-diol Diacetate (12), and 2,6-Dimethyl-2(E),6(E)-octadien-1,8-diol Dibutyrate (16). Geraniol (**I**) was converted to geranyl acetate (**II**), which was then oxidized using selenium dioxide in aqueous dioxane as described for linalool (Hirata et al., 1981), to give the previously known compound (Meinwald and Opheim, 1973), 8-oxo-3,7-dimethyl-2(*E*),6(*E*)-octadienyl acetate (**10**) (Figure 2); EI-MS [*m/z* (%)]: 150 (12%), 135 (9), 121 (10), 107 (5), 95 (3), 93 (6), 91 (3), 85 (16), 84 (40), 82 (9), 68 (4), 67 (8), 65 (3), 55 (11), 53 (7), 43

(100), 41 (12), 39 (10); CI-MS (NH_3) [*m/z* (%)]: 245 [M + $(NH_3)_2H$, 22%], 228 (M + NH_4, 100); ^1H NMR ($CDCl_3$): δ 1.75, broad s, two Me; 2.05, s, Ac Me; 2.24, t, J = 7.5 Hz, two H5; 2.51, dt (app. q), two H4; 4.60, d, J = 6.9 Hz, two H8; 5.39, tq, J = 6.9 and 2.4 Hz, H3; 6.45, tq, J = 6.9 and 2.4 Hz, H7 (H3 and H7 may be reversed); 9.39, s, CHO; literature (Meinwald and Opheim, 1973) (CCl_4): δ 1.75, s, 6H; 1.97, s, 3H; 2.35, m, 4H; 4.55, d, J = 7 Hz, 2H; 5.40, broad t, J = 7 Hz, 1H; 6.42, broad t, J = 6 Hz, 1H; 9.38, s, 1H.

Synthetic **10** was reduced with sodium borohydride to 8-hydroxy-3,7-dimethyl-2(*E*),6(*E*)-octadienyl acetate (**III**). Acetylation of **III** with acetic anhydride gave 2,6-dimethyl-2(*E*),6(*E*)-octadien-1,8-diol diacetate (**12**); EI-MS [*m/z* (%)]: 212 (0.2%), 195 (0.5), 152 (2), 135 (5), 134 (21), 127 (6), 126 (8), 119 (15), 107 (2), 105 (3), 93 (5), 92 (3), 91 (3), 85 (12), 84 (30), 79 (4), 68 (12), 67 (14), 55 (4), 53 (5), 43 (100), 41 (10), 39 (5); CI-MS (NH_3) [*m/z* (%)]: 526 (2M + NH_4, 8%), 289 [M + $(NH_3)_2H$, 15], 272 (M + NH_4, 100); ^1H NMR ($CDCl_3$): δ 1.66, s, Me9; 1.71, s, Me10; 2.06, s, Ac Me; 2.07, s, Ac Me; 4.45, s, two H1; 4.59, d, J = 7.2 Hz, two H8; 5.35, tq, J = 7.1 and 2.1, H7; 5.44, tq, J = 6.9 and 1.2, H3.

Saponification of **12** gave 9-hydroxygeraniol (**9**); EI-MS [*m/z* (%)]: 137 (6%), 134 (4), 121 (11), 119 (7), 115 (7), 109 (8), 107 (6), 95 (10), 94 (12), 93 (9), 84 (38), 83 (13), 81 (14), 71 (17), 69 (15), 68 (61), 67 (32), 57 (20), 55 (25), 53 (18), 43 (100), 41 (57), 39 (21); CI-MS (NH_3) [*m/z* (%)]: 205 [M + $(NH_3)_2H$, 8%], 188 (M + NH_4, 100), 170 (M + NH_4 − H_2O, 6); CI-MS (ND_3) [*m/z* (%)]: 214 [M + $(ND_3)_2D$ − 2H + 2D, 15%], 194 (M + ND_4 − 2H + 2D, 100).

Treatment of **9** with butyryl chloride gave 2,6-dimethyl-2(*E*),6(*E*)-octadien-1,8-diol dibutyrate (**16**), EI-MS [*m/z* (%)]: 155 (3), 154 (5), 135 (7), 134 (19), 119 (16), 107 (3), 106 (3), 93 (6), 92 (4), 84 (8), 79 (3), 71 (100), 68 (9), 67 (9), 55 (4), 53 (3), 43 (41), 41 (12); CI-MS (NH_3) [*m/z* (%)]: 345 (M + $(NH_3)_2H$, 2%], 328 (M + NH_4, 100).

*8-Hydroxy-2,6-dimethyl-2(*E*),6(*E*)-octadienyl Acetate (**11**), 2,6-Dimethyl-2(*E*),6(*E*)-octadien-1,8-diol 1-Butyrate (9-Hydroxygeranyl Butyrate) (**13**), and 2,6-Dimethyl-2(*E*),6(*E*)-octadien-1,8-diol 1-Acetate 8-Butyrate (**15**).* 8-Hydroxy-2,6-dimethyl-2(*E*),6(*E*)-octadienyl acetate (**11**) was prepared from the *t*-butyldimethylsilyl ether of geraniol (**IV**) by a parallel series of reactions consisting of sequential selenium dioxide oxidation (**V**), sodium borohydride reduction (**VI**), esterification with acetic anhydride (**VII**), and desilylation with 1 M tetrabutylammonium fluoride in THF for 1 hr at room temperature; EI-MS of **11** [*m/z* (%)]: 152 (1%), 137 (2), 134 (8), 123 (2), 121 (40), 119 (6), 109 (3), 107 (2), 94 (5), 93 (6), 85 (8), 84 (33), 79 (5), 69 (6), 68 (36), 67 (19), 57 (4), 55 (8), 53 (6), 43 (100), 41 (18), 39 (8); CI-MS (NH_3) [*m/z* (%)]: 247 [M

+ $(NH_3)_2H$, 9%], 230 (M + NH_4, 100); CI-MS (ND_3) [*m/z* (%)]: 255 [M + $(ND_3)_2D$ − H + D, 15%], 235 (M + ND_4 − H + D, 100).

Selenium dioxide oxidation of geranyl butyrate (**VIII**) by the procedure used to prepare **10** (Hirata et al., 1981) gave the 8-butyrate of 8-hydroxy-2,6-dimethyl-2(*E*),6(*E*)-octadienal (**IX**). Sodium borohydride reduction gave 9-hydroxygeranyl butyrate (**13**); EI-MS [*m/z* (%)]: 154 (2%), 152 (2), 137 (4), 135 (3), 134 (14), 123 (4), 121 (9), 119 (10), 109 (4), 107 (4), 95 (8), 94 (11), 93 (10), 84 (44), 81 (7), 79 (8), 71 (100), 69 (11), 68 (52), 67 (22), 57 (6), 55 (11), 53 (7), 43 (84), 41 (31), 39 (10); CI-MS (NH_3) [*m/z* (%)]: 275 [M + $(NH_3)_2H$, 6%], 258 (M + NH_4, 100); (ND_4) *m/z* 283 [M + $(ND_3)_2D$ − H + D, 8%], 263 (M + ND_4 − H + D, 100).

*2,6-Dimethyl-2(E),6(E)-octadien-1,8-diol 1-Acetate 8-Propionate (**14**).* EI-MS [*m/z* (%)]: 209 (0.6%), 152 (2), 141 (3), 140 (5), 135 (7), 134 (25), 127 (4), 126 (5), 119 (20), 105 (4), 93 (5), 92 (2), 85 (10), 84 (28), 81 (2), 69 (11), 67 (15), 57 (100), 55 (5), 53 (6), 43 (72), 41 (8), 39 (5); CI-MS (NH_3) [*m/z* (%)]: 303 [M + $(NH_3)_2H$, 3%], 286 (M + NH_4, 100).

Acetylation of **13** gave 2,6-dimethyl-2(*E*),6(*E*)-octadien-1,8-diol 1-acetate 8-butyrate (**15**); EI-MS [*m/z* (%)]: 223 (0.5%), 195 (0.3), 155 (2), 154 (3), 152 (2), 135 (7), 134 (25), 127 (4), 126 (5), 121 (3), 119 (20), 107 (3), 105 (4), 93 (7), 92 (4), 85 (7), 84 (22), 79 (4), 71 (84), 68 (13), 67 (14), 55 (5), 53 (5), 43 (100), 41 (16), 39 (5); CI-MS (NH_3) [*m/z* (%)]: 317 [M + $(NH_3)_2H$, 3%], 300 (M + NH_4, 100).

RESULTS AND DISCUSSION

Oechalia adults reared from eggs of the female collected in November at the Maroochy Horticultural Research Station, Nambour, Queensland, did not exhibit sexually dimorphic dorsal abdominal glands after two to three weeks in captivity and, similarly, the glands of males collected April 26, 1994, in alfalfa fields near Gatton, Queensland, were not noticeably larger than those of females. However, the dorsal abdominal glands of *O. schellenbergii* males collected March 19, 1991, in alfalfa at the Gatton site were roughly 50 times larger than those of females. Two 150-μl dichloromethane extracts of the enlarged glands from groups of six and seven of these wild adult males provided sufficient material for characterization of the secretion.

Sixteen components were identified and account for 96% of the total detectable components eluting from the column. Mass spectral data for components **1, 3,** and **6** indicated that these compounds were identical to previously known, common exocrine constituents from Heteroptera (Aldrich, 1988), and comparisons to authentic standards verified these structures as (*E*)-2-hexenal, (*E*)-2-

hexenyl acetate, and (*E*)-2-hexenyl butyrate, respectively. The mass spectrum of minor component **8** matched that of the trimethylester of isocitric acid, and coeluted with the synthetic compound. The mass spectra of the remaining components of the secretion were not identical to available published spectra (Hewlett-Packard MS library). CI-MS (NH_3 and ND_3 CI-MS) established that **2, 8, 11,** and **13** possess one exchangeable proton each, **9** contains two exchangeable protons, while the remaining numbered components lack exchangeable protons.

Compound **2** (MW 114) appeared to be an unsaturated alcohol, but eluted prior to (*E*)-2-heptenol, suggesting that **2** is a branched, monounsaturated alcohol. The CI-MS of **4** indicated MW 156, while in the EI-MS there was a base peak at *m/z* 43 plus prominent ions at *m/z* 81 and 96, which were the same as those of **2,** suggesting that **4** may be the acetate ester of **2.** Hydrogenation of a portion of the insect secretion and comparison of the EI-MS of the saturated product of **2** with published spectra of various C-7 alcohols (Baumeister et al., 1991) suggested that it was a primary alcohol with a methyl branch at either the 2 or 3 position. Branching at the 2 position was eliminated as a possible structure feature because comparison of the MS of saturated **2** to the MS of 2-methyl-1-hexanol prepared from 2-methyl-2-(*E*)-hexenoic acid showed they were different. However, a commercial standard of 3-methyl-1-hexanol coeluted with and gave a mass spectrum identical to that of saturated **2.** Likewise, the hydrogenation product of **4** coeluted with and gave a spectrum that was identical to the EI-MS of synthetic 3-methylhexyl acetate.

The position of unsaturation in **2** and **4** was determined by synthesis and NMR analysis of synthetic **4** (see above); synthetic **2** and **4** were identical to the insect-produced materials by mass spectrometry, and coeluted by GC with the respective insect-derived materials. Treatment of **2** with butyryl chloride gave synthetic **7,** which was identical to insect-derived **7.** We infer that **5** is the corresponding propionate, 3-methylenehexyl propionate.

The CI-MS of **12** indicated a MW of 254, and the EI-MS exhibited an exceptionally abundant base peak at *m/z* 43. The greater abundance of the *m/z* 43 ion in the EI-MS of **12** relative to that for **4** suggested the possibility of a diacetate ester of a $C_{10}H_{18}O_2$ diol. This possibility was strengthened by the observation that the EI-MS of **14** and **15** showed less abundant *m/z* 43 ions with the addition of ions at *m/z* 57 and 71, respectively, suggesting that **14** and **15** (MW = 268 and 282, respectively) could be monopropionate and monobutyrate (or monoisobutyrate) homologs of **12.** Earlier discovery of 8-hydroxylinalool from an exocrine secretion of another true bug (Aldrich et al., 1990) raised the prospect that **12** could be the diacetate of 8-hydroxylinalool. Synthetic 8-hydroxylinalyl diacetate [prepared from an earlier diol sample (Aldrich et al., 1990)] eluted before **12** by GC, but the similarity of its EI-MS to that of **12** prompted us to prepare the corresponding diacetate derived from geraniol.

Geranyl acetate was oxidized with SeO_2 to give 8-oxo-3,7-dimethyl-2(*E*),6(*E*)-octadienyl acetate, which proved identical to insect-derived **10.** Synthetic **10** was reduced with sodium borohydride to 8-hydroxy-3,7-dimethyl-2(*E*),6(*E*)-octadienyl acetate (**III**), whose mass spectrum and GC retention time were very similar, but not identical, to those of the insect material (**11**). Acetylation of **III** gave diacetate **12,** which was indistinguishable from the insect-derived **12,** and saponification gave a diol whose properties were identical to those of insect-derived **9.** Treatment of **9** with butyryl chloride gave dibutyrate **16.** These conclusions suggested that the correct monoacetate should be the geranyl diol acetylated at the opposite terminus. Consistent with this conclusion, **11** was synthesized (see above) and was found to be identical to the insect-produced material.

Similarly, treatment of monoacetate **III** with butyryl chloride gave monoacetate, monobutyrate **X** whose retention times and mass spectra were very similar, but not identical, to those of the natural monoacetate, monobutyrate, indicating **15** as the correct isomer. Selenium dioxide oxidation of geranyl butyrate gave the 8-butyrate of 8-hydroxy-2,6-dimethyl-2(*E*),6(*E*)-octadienal (**IX**). Sodium borohydride reduction gave monobutyrate **13,** identical to insect-derived material, and acetylation gave the monoacetate, monobutyrate **15,** which was identical to insect-derived **15.** By analogy, we infer that the insect-produced monoacetate, monopropionate isomer is 2,6-dimethyl-2(*E*),6(*E*)-octadien-1,8-diol 1-acetate 8-propionate (**14**).

In summary, adult *Oechalia schellenbergii* males have dorsal abdominal glands that are much larger than those of females, at least during part of their life cycle, and these glands are homologous to those of other predatory bugs whose male-specific secretions have proven to be attractant pheromones (Aldrich, 1988, 1995). Thus, it is likely that the secretion from the enlarged glands of *Oechalia* males also serves as a pheromone.

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MAMMALIAN EXOCRINE SECRETIONS: X. CONSTITUENTS OF PREORBITAL SECRETION OF GRYSBOK, *Raphicerus melanotis*

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Abstract—Using electron impact and chemical ionization mass spectrometry with methane and nitric oxide as reactant gases in conjunction with mass spectral analysis of the dimethyl disulfide derivatives of unsaturated constituents, 51 compounds have been identified in addition to the compounds previously found in the preorbital secretion of the grysbok *Raphicerus melanotis*. The compounds identified are saturated, monounsaturated, and diunsaturated alcohols and formates, saturated and monounsaturated aldehydes, saturated carboxylic acids, γ- and δ-lactones, alkyl methyl sulfides, and β-hydroxyalkyl acetates. All of these compounds have unbranched structures. The constituent previously incorrectly identified as (Z)-5-tetradecen-1-ol, was found to consist of the coeluting (Z)-7- and (Z)-8-tetradecenols.

Key Words—*Raphicerus melanotis*, mammalian semiochemicals, mammalian pheromones, exocrine secretion, preorbital secretion, double bond localization, mass spectrometry.

INTRODUCTION

The Cape grysbok, *Raphicerus melanotis*, is a small, squat antelope characterized by rufous brown upperparts abundantly flecked with white hairs and underparts with a lighter brown color. The incidence of white hairs diminishes down the sides and legs. The tail is short and the grey–brown ears are proportionately large with white hairs on the inside. The average shoulder height is about 54 cm and the average mass is about 10 kg. Only rams have short, smooth, slightly

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back-angled horns with an average length of about 8 cm (Stuart and Stuart, 1990). The rufous brown upperparts flecked with white distinguish it from the steenbok, as do the brown underparts. The Cape grysbok is similar to Sharp's grysbok, *Raphicerus sharpei*, in appearance, but their distribution ranges do not overlap (Stuart and Stuart, 1990).

The distribution of the grysbok is restricted to a narrow belt along the southwestern and southern Cape coast and adjacent interior of South Africa. An inhabitant of relatively thick scrub–bush, it is almost entirely restricted to the "fynbos" vegetation of the Cape. It is mainly a nocturnal animal but is active in the early morning and late afternoon if not disturbed, or on overcast and cool days. Grysbok are usually single except when mating or when ewes are tending lambs. Although lambs may be dropped at any time of the year, most are born from September to December. A single lamb is born after a gestation period of approximately 180 days. The grysbok is considered to be a nuisance in vineyards as it grazes on the young grapes and terminal buds.

The grysbok belongs to the order Artiodactyla, the family Bovidae and the subfamily Antilopinae. Members of this subfamily, also known as dwarf antelope, vary considerably in appearance and habitat. The pygmy antelope, *Neotragus batesi*, for instance, inhabits the dense forests of Central Africa, whereas the oribi, *Ourebia ourebi*, is found on open grass plains near water. The klipspringer, *Oreotragus oreotragus*, prefers mountainous territory and the dik-dik, *Madoqua kirkii*, is found in arid bushveld regions. The only similarities between members of this subfamily are their size and their well-developed exocrine glands, especially the preorbital glands, which are used for territorial marking (Macdonald, 1984). Territoriality is important to these animals as it is essential for them to have the necessary knowledge regarding food supplies in their environment and to eliminate any competition with regard to their food supply. Feeding is an important factor because their metabolic processes are rapid as a result of their small body size. Consequently, in relation to their body size, they need more nutritious food than larger animals (Macdonald, 1984). As the grysbok is found in areas with dense vegetation, olfactory communication is expected to be an important means of communication. Both sexes possess active preorbital glands, the secretions of which consist of a black aqueous emulsion of organic compounds. Grysbok rams are solitary and territorial and mark territories with the preorbital secretion (Novellie et al., 1984). Although no qualitative and only small, probably insignificant, differences were found between the male and female secretions (Le Roux, 1980), marking activity is only rarely observed in females.

In a previous study (Le Roux, 1980; Burger et al., 1981a) 34 of the constituents of the preorbital secretion of the grysbok were identified. Due to the uninformative mass spectra of long-chain, unbranched compounds, only a limited number of these constituents were identified solely by means of mass spec-

trometry. Some of the major representative constituents were therefore isolated for NMR analysis using preparative gas chromatography. This entailed the collection of 1000 gland equivalents of the secretion from five animals over many months. In spite of an enormous investment in time, only 18 constituents of the secretion were isolated in sufficient quantities for ^{1}H NMR analysis. Of five of these constituents ^{13}C NMR spectra could be obtained. The position of the double bonds in some of these compounds was determined by microozonolysis. The *Z* configuration was assigned to unsaturated compounds on the available ^{1}H and ^{13}C NMR data.

The availability of more efficient capillary columns, and new methods for the determination of the position of double bonds in long-chain compounds, led to the decision to reinvestigate the preorbital secretion of the grysbok. In this communication we report the confirmation of the structures of 27 of the compounds identified in the previous investigation and the identification of another 51 constituents of the secretion.

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. Syringes, stainless-steel needles, etc., were cleaned by rinsing with this solvent.

Analytical Methods. Gas chromatographic (GC) analyses were carried out with Carlo Erba 4200 and 5300 gas chromatographs equipped with flame ionization detectors, Grob split-splitless injectors, and glass columns coated by the Laboratory for Ecological Chemistry with a 0.25-mm film of the apolar stationary phases, PS-089-OH, a silanol-terminated, 95%-dimethyl–5%-diphenylsiloxane copolymer, and OV-1701-OH. Helium was used as carrier gas at a linear velocity of 28.6 cm/sec at 40°C. The flame ionization detector was operated at 280°C and the injector was normally used at 220°C. In analyses of material dissolved in dichloromethane, the samples were injected in the split mode, and the volatiles entering the column were thermally focused at ca. 30°C, and subsequently analyzed using a temperature program of 2°C/min from 40°C to 260°C (hold). In analyses in which solventless sample introduction was used, cryotrapping with Dry Ice was employed. Quantification was done with Barspec software and a 386 personal computer.

Electron impact (EI) mass spectra were recorded at 70 eV on a Carlo Erba QMD 1000 gas chromatograph–mass spectrometer (GC-MS system), using the

columns and conditions described above. The QMD 1000 consists of a Carlo Erba 5300 gas chromatograph with a line-of-sight interface to a VG Analytical Trio 1 quadrupole mass spectrometer. An interface temperature of 250°C was used. The ion source temperature was set at 200°C and the pressure in the source housing was ca. 2×10^{-5} torr at a column temperature of 40°C, decreasing to ca. 1×10^{-5} torr towards the end of the temperature program.

Chemical ionization (CI) mass spectra were obtained with methane and nitric oxide as reactant gases. With methane as reactant gas, spectra were recorded at 75 eV and a source temperature of 150°C. The flow of methane into the ion volume of the source was regulated with a fine metering valve to obtain equally abundant *m/z* 17 and 29 plasma ions. With NO as reactant gas, an iridium filament coated with thorium oxide was used. Spectra were recorded at 75 eV and a source temperature of ca. 80°C. The source pressure was adjusted to obtain a maximum abundance of the *m/z* 60 plasma ion. With isobutane as reactant gas, optimal tuning was reached by adjusting the source pressure to produce the *m/z* 39, 43, and 57 plasma ions in a ratio of 1:4:6. All three reactant gases required a source housing pressure of ca. 8×10^{-5} torr for optimal tuning.

Sample Collection and Preparation. Preorbital secretion was collected with a tubular PTFE scoop from animals kept in pens (10 × 10 m) at the Tygerberg Zoo. The scoop, with an inside diameter of 5 mm, was furnished with a PTFE plunger with which the collected material could be ejected into a 300-μl or 1-ml Reacti-Vial.

The organic volatiles were extracted from the secretion by stirring collected material with dichloromethane (ca. 100 μl) using a thin (0.5 mm) glass rod to produce a homogeneous suspension that was centrifuged at 2000–3000 rpm for about 15 min to separate solid material, the dichloromethane extract, and a small quantity of water and mucus. The dichloromethane extract was removed from underneath the supernatant water and mucus layer with a 100-μl syringe, transferred to a Reacti-Vial, and stored at −30°C. Most extracts could be used without subsequent evaporation of the solvent. If more concentrated solutions were required, the solvent was removed in a slow stream of purified (activated charcoal) nitrogen.

Dimethyl disulfide (DMDS) derivatization of the organic material extracted from the secretion was carried out according to the method described by Vincenti et al. (1987). A 10-μl aliquot of a dichloromethane extract of the preorbital secretion was concentrated in a 1-ml Reacti-Vial using a slow stream of purified nitrogen, the residual material dissolved in dimethyl disulfide (50 μl), and treated with a solution of iodine (300 μg) in carbon disulfide (50 μl). The Reacti-Vial was sealed using a PTFE-faced rubber septum and the reaction mixture left at 60°C for 40 hr in the oven of a gas chromatograph. The reaction was quenched with aqueous sodium thiosulfate solution (5%). The organic and aqueous layers

were separated by centrifuging for a few minutes at 2000 rpm after which the organic layer was transferred to another Reacti-Vial. The solution was concentrated to 5 μl for GC-MS analysis.

Reference Compounds. Some of the compounds identified in the preorbital secretion of the grysbok are commercially available, while others were available from previous research projects in this series. The following compounds were synthesized during the present investigation.

Heptadecanyl formate was synthesized by the uncatalyzed reaction of 1-heptadecanol (50 mg) with an excess of absolute formic acid (100 μl). The reaction mixture was heated overnight at 80°C in a Reacti-Vial, after which the excess formic acid was removed in vacuo to give pure (GC) heptadecan-1-yl formate. ^{13}C: δ ($CDCl_3$) 161.18 (s, C-1'), 64.14 (t, C-1), 31.95 (t, C-15), 29.21–29.71 (11C, t, C-4–C-14), 28.54 (t, C-2), 25.84 (t, C-3), 22.71 (t, C-16), 14.12 (q, C-17).

Octadecan-4-olide was synthesized by the Mn(III) acetate-catalyzed free radical coupling of hexadec-1-ene and acetic acid (Heiba et al., 1983). $KMnO_4$ (1.09 g; 7 mmol) was added to a stirred solution of Mn(II) acetate (7.07 g; 29 mmol) in acetic acid (40 ml). After an initial increase in the temperature, the reaction mixture was allowed to cool to 90°C. The temperature increased on slow addition of acetic anhydride (10 ml) and the reaction mixture was again allowed to cool to 90°C, after which hexadec-1-ene (5.01 g; 23 mmol) was added to the mixture. This was immediately followed by the addition of anhydrous sodium acetate (16.7 g, 204 mmol). The reaction mixture was refluxed for 2 hr, diluted with a large volume of water (120 ml), the γ-lactone extracted with benzene, and the extract dried on anhydrous Na_2SO_4. Removal of the solvent and bulb-to-bulb distillation gave the pure (GC) octadecan-4-olide (4.95 g, 78%), bp 235°C/0.015 torr (air bath temperature). ^{13}C: δ ($CDCl_3$) 177.24 (s, C-1), 81.06 (d, C-4), 35.64 (t, C-2), 31.97 (t, C-16), 29.40–29.74 (10C, t, C-6–C-15), 28.04 (t, C-3), 25.27 (t, C-5), 22.72 (t, C-17), 14.12 (q, C-18).

Hexadecan-5-olide was synthesized by oxymercuration of tridec-1-ene, followed by free radical coupling of the product with acrylonitrile, and hydrolysis of the resulting 5-hydroxyhexadecane-1-nitrile according to the method of Giese et al. (1984). Mercury(II) acetate (4.35 g; 13.6 mmol) and tridec-1-ene (2.49 g; 13.6 mmol) were added to a solution of THF (35 ml) in water (15 ml), and the reaction mixture was stirred for 1 hr. Acrylonitrile (2.16 g; 40.7 mmol), sodium hydroxide solution (2 M, 15 ml), and sodium borohydride (1.03 g; 27.2 mmol) dissolved in sodium hydroxide solution (2 M, 55 ml) were added to the reaction mixture, which was stirred for a further 30 min. The organic material was extracted with dichloromethane. Removal of the solvent gave the crude hydroxynitrile, which was saponified by refluxing it with sodium hydroxide (2 M, 40 ml) for 4 hr. Unsaponified material was extracted with ether, after which the water layer was acidified with sulfuric acid (2 M) and the δ-lactone extracted

with ether, the ether extract dried on anhydrous Na_2SO_4, and the solvent removed in vacuo. Bulb-to-bulb distillation gave the required hexadecan-5-olide (2.36 g, 68%), bp 175°C/0.02 torr (air bath temperature). ^{13}C: δ ($CDCl_3$) 171.97(s, C-1), 80.61(d, C-5), 35.88(t, C-2), 31.94(t, C-14), 29.37–29.69(8C, t, C-6–C-13), 27.83(t, C-4), 24.96(t, C-6), 22.70(t, C-15), 18.53(t, C-3), 14.11(q, C-16).

RESULTS AND DISCUSSION

A typical total ion chromatogram of an extract of the preorbital secretion is shown in Figure 1. Tentative identification of a number of the volatile organic constituents was based on a comparison of their mass spectra with those in NBS and Wiley mass spectra libraries. Further structural information on many of the volatile constituents of the secretion was furnished by their chemical ionization mass spectra obtained with methane and nitric oxide as reactant gases. Information on the position of the double bond(s) in some of the unsaturated constituents was obtained from their CI(NO) mass spectra (Malosse and Einhorn, 1990) and the EI mass spectra of their dimethyl disulfide derivatives (Buser et al., 1983; Carlson et al., 1989; Vincenti et al., 1987).

The most salient aspects of the identification of most of the compound types present in preorbital secretions based on their EI, CI(CH_4) and CI(NO) spectra have been discussed in considerable detail in the previous communication in this series (Mo et al., 1995) and will not be repeated here. However, since mass spectral information on long-chain compounds is not readily available, the relevant information for some of the constituents identified in the secretion, is given in Tables 1 to 6. The diagnostic ions in the EI mass spectra of the DMDS derivatives of the mono- and diunsaturated compounds that were used to determine the position of the double bonds in these compounds, are summarized in Tables 7 and 8.

In contrast to the preorbital secretion of the oribi, which contains compounds having a finite chain length (Mo et al., 1995), the preorbital secretion of the grysbok appears to contain large concentrations of compounds having chain lengths beyond those identified in the present investigation. These compounds have very low vapor pressures and, if the frequency of territorial marking in the grysbok is taken into account, it seems unlikely that these long-chain compounds could be essential ingredients of the territorial marking pheromone of the animal. They may, however, constitute a controlled-release medium for the more volatile constituents of the secretion. A consequence of the presence of these long-chain compounds in the secretion is that capillary columns coated with highly polar and thermally less stable stationary phases such as Carbowax,

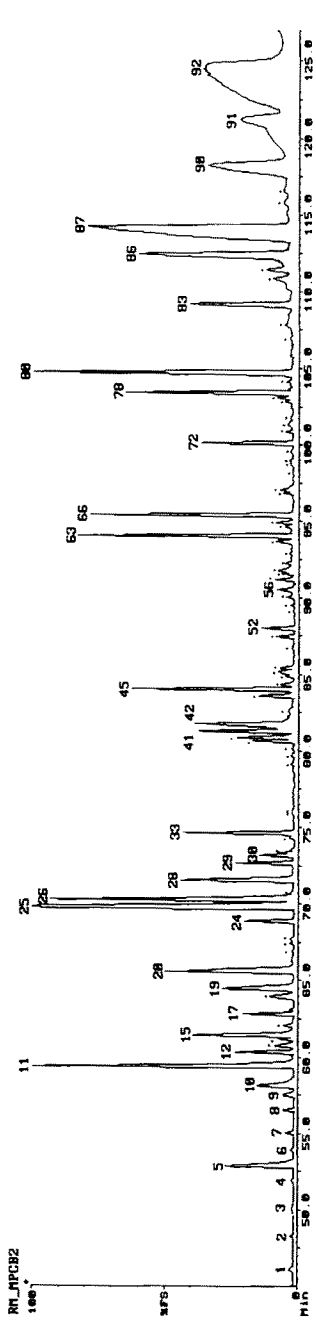


FIG. 1. Total ion chromatogram of an extract of the preorbital secretion of the grysbok.

TABLE I. DIAGNOSTIC IONS IN EI, CI(CH_4), AND CI(NO) MASS SPECTRA OF 1-ALKANOLS PRESENT IN PREORBITAL SECRETION OF GRYSBOK

No. in Figure 1	1-Alkanol	Ion (%, normalized abundance)								
		EI		CI(CH_4)			CI(NO)			
		$(M - 18)^+$	$(M - 46)^+$	M^+	$(M - 1)^+$	$(M - 17)^+$	$(M + 28)^+$	M^+	$(M - 1)^+$	$(M - 3)^+$
1	1-Undecanol	154[a]	126 (46)	172 (4)	171 (76)	155 (100)	200 (100)	172 (5)	171 (20)	169 (90)
5	1-Dodecanol	168 (0.4)	140 (5)	186 (10)	185 (77)	169 (100)	214 (97)	186 (3)	185 (22)	183 (100)
11	1-Tridecanol	182 (0.4)	154 (4)	200 (11)	199 (80)	183 (100)	228 (100)	200 (3)	199 (22)	197 (92)
20	1-Tetradecanol	196 (0.4)	168 (4)	214 (14)	213 (92)	197 (100)	242 (100)	214 (4)	213 (30)	211 (75)
28	1-Pentadecanol	210 (0.4)	182 (3)	228 (16)	227 (100)	211 (98)	256 (100)	228 (4)	227 (30)	225 (76)

[a] Not detected.

TABLE 2. DIAGNOSTIC IONS IN EI, CI(CH_4), AND CI(NO) MASS SPECTRA OF 1-ALKANYL FORMATES PRESENT IN PREORBITAL SECRETION OF GRYSBOK

No. in Figure 1	Formate	Diagnostic ion (%, normalized abundance)							
		EI		CI(CH_4)				CI(NO)	
		$(M - 46)^+$	$(M - 74)^+$	M^+	$(M - 1)^+$	$(M - 29)^+$	$(M - 45)^+$	$(M + 30)^+$	$(M - 1)^+$
3	Undecyl	154[a]	126 (12)	200[a]	199[a]	171[a]	155 (100)	230 (100)	199[a]
8	Dodecyl	168 (2)	140 (8)	214 (1)	213 (5)	185[a]	169 (100)	244 (100)	213[a]
17	Tridecyl	182 (2)	154 (6)	228 (1)	227 (6)	199 (2)	183 (100)	258 (100)	227 (1)
24	Tetradecyl	196 (2)	168 (5)	242 (1)	241 (7)	213 (4)	197 (100)	272 (100)	241 (6)
33	Pentadecyl	210 (1)	182 (3)	256 (1)	255 (10)	227 (3)	211 (100)	286 (100)	255 (3)
38	Hexadecyl	224[a]	196 (1)	270 (2)	269 (13)	241 (6)	225 (100)	300 (100)	269[a]
49	Heptadecyl	238[a]	210 (2)	284 (3)	283 (15)	255 (8)	239 (100)	314 (100)	283 (6)
56	Octadecyl	252[a]	224 (2)	298 (3)	297 (20)	269 (8)	253 (100)	328 (100)	297 (2)
66	Nonadecyl	266[a]	238 (2)	312 (4)	311 (23)	283 (6)	267 (100)	342 (100)	311 (2)
72	Icosyl	280[a]	252 (1)	326 (4)	325 (20)	297 (10)	281 (100)	356 (100)	325 (12)
80	Henicosyl	294[a]	266 (1)	340 (7)	339 (28)	311 (10)	295 (100)	370 (100)	339 (15)
83	Docosyl	308[a]	280[a]	354 (10)	253 (30)	325 (11)	309 (100)	384 (100)	353 (4)
87	Tricosyl	322[a]	294[a]	368 (10)	367 (32)	339 (17)	323 (100)		
90	Tetracosyl	336[a]	308[a]	382 (15)	381 (60)	353 (22)	337 (100)		
92	Pentacosyl	350[a]	322[a]	396 (7)	395 (55)	367 (15)	351 (100)		

[a]Not detected.

TABLE 3. DIAGNOSTIC IONS IN EI, CI(CH_4), AND CI(NO) MASS

No. in Figure 1	Alkenol	EI		CI(CH_4)					
		M^+	$(M-18)^+$	$(M-46)^+$	$(M+1)^+$	M^+	$(M-1)^+$	$(M-17)^+$	$(M-18)^+$
4	6-Dodecen-1-ol	184[a]	166[a]	138 (10)	185 (7)	184 (2)	183 (9)	167[a]	166 (15)
10[b]	6-Tridecen-1-ol 7-Tridecen-1-ol	198[a]	180 (3)	152 (4)	199 (10)	198 (2)	197 (13)	181 (4)	180 (19)
19[b]	8-Tetradecen-1-ol 7-Tetradecen-1-ol	212[a]	194 (2)	166 (1)	213 (12)	212 (2)	211 (13)	195 (5)	194 (17)
26[b]	8-Pentadecen-1-ol 6-Pentadecen-1-ol	226[a]	208 (2)	180 (1)	227 (16)	226 (3)	225 (15)	209 (4)	208 (21)
41	8-Heptadecen-1-ol	254[a]	236 (2)	208 (1)	255 (16)	254 (3)	253 (19)	237 (4)	236 (25)
58	10-Nonadecen-1-ol	282[a]	264[a]	236[a]	283 (19)	282 (3)	281 (20)	265 (7)	264 (28)

[a] Not detected.
[b] Coeluting isomers are given in the order in which their DMDS derivatives are eluted.

could not be employed in GC and GC-MS analyses, as these phases cannot survive repeated high-temperature conditioning. Limited use was made of capillary columns coated with OV-1701-OH, but the apolar PS-089-OH column produced the best results, although some of the isomeric alkenols were found to coelute from this column. Fortunately the DMDS derivatives of these compounds were separated on the apolar column and could therefore be unequivocally identified.

Probably because it has a relatively large ion volume, the CI ion source of the QMD 1000 mass spectrometer gives poor CI spectra with a build-up of the baseline towards the end of the total ion chromatogram. It was nevertheless possible to obtain useful information from the CI(CH_4) spectra of some of the more volatile constituents of the secretion. The use of NO as CI reactant gas deserves mention. The CI(NO) mass spectra furnish information from which the molecular mass of a compound, in many cases the compound type, and the position of the double bond in monounsaturated compounds can be derived (Hunt et al., 1982). Due to the previously noted problems with the CI source, ions furnishing information on the position of the double bond in unsaturated compounds could be detected only in the spectra of compounds that were present in relatively high concentrations and/or were well separated from the other constituents of the secretion. The results obtained in these analyses nevertheless illustrated the potential of NO as CI reactant gas.

SPECTRA OF ALKENOLS PRESENT IN PREORBITAL SECRETION OF GRYSBOK

Diagnostic ion (%, normalized abundance)

						CI(NO)		
$(M-19)^+$	$(M+28)^+$	M^+	$(M-1)^+$	$(M-2)^+$	$(M-3)^+$	$(M-19)^+$	$[CH_3(CH_2)_nCO]^+$	$[CH_3(CH_2)_nCH{=}N\text{-}OH]^+$
165 (12)	212[a]	184[a]	183[a]	182[a]	181[a]	165[a]		
179 (15)	226[a]	198[a]	197 (57)	196 (2)	195 (6)	179[a]	99 (17)	158 (23)
193 (12)	240 (12)	212 (14)	211 (100)	210 (40)	209 (15)	193[a]		158 (37)
207 (13)	254 (29)	226 (17)	225 (100)	224 (37)	223 (40)	207 (41)	141 (10)	158 (24)
235 (13)	282 (95)	254 (20)	253 (100)	252[a]	251[a]	235[a]		
263 (14)	310 (7)	282 (1)	281 (22)	280 (15)	279 (3)	263 (28)		

Only two saturated and one unsaturated aldehyde are present in low concentrations in the preorbital secretion of the grysbok, whereas the oribi's secretion contains several saturated, monounsaturated, and diunsaturated aldehydes. The possibility was considered that this difference could be the result of autoxidation of the aldehydes taking place much more readily in the open preorbital cavity of the grysbok than in the deeper preorbital structure of the oribi. However, since neither the secretion of the oribi nor that of the grysbok contains detectable amounts of carboxylic acids and aldehydes having the same chain lengths, this does not seem to be a tenable hypothesis.

The EI mass spectra of components 43, 52, 61, 69, and 81 all have a base peak of high relative abundance at m/z 85. These compounds were identified as pentadecan-4-olide, hexadecan-4-olide, heptadecan-4-olide, octadecan-4-olide, and icosan-4-olide, respectively using, among others, data obtained from their CI mass spectra.

In addition to these compounds having a base peak at m/z 85 in their EI mass spectra, the preorbital secretion contains component 54 with an equally abundant base peak at m/z 99 in its EI mass spectrum. Taking the unbranched structures of the other constituents of the preorbital secretion into consideration, the possibility that this component could be an alkan-5-olide or δ-lactone, was considered. The molecular ion is not observed in its EI mass spectrum, but the CI(CH_4) mass spectrum affords conclusive evidence that the compound has a

TABLE 4. DIAGNOSTIC IONS IN EI, CI(CH_4), AND CI(NO) MASS SPECTRA OF ALKENYL FORMATES PRESENT IN PREORBITAL SECRETION OF GRYSBOK

No. in Figure 1	Formates	Diagnostic ion (%, normalized abundance)												
		EI		CI(CH_4)					CI(NO)					
		M$^+$	(M − 46)$^+$	(M + 1)$^+$	(M − 1)$^+$	(M − 29)$^+$	(M − 45)$^+$	(M − 46)$^+$	(M + 30)$^+$	M$^+$	(M − 1)$^+$	(M − 46)$^+$	A$^+$ = [$(CH_2)_n$-CO]$^+$	B$^+$ = [OC-$(CH_2)_m$-$OOCH$]$^+$
16	7-Tridecenyl	226[a]	180[a]	227 (2)	225 (1)	197 (1)	181 (7)	180 (24)						
22	7-Tetradecenyl	240[a]	194 (6)	241 (1)	239 (1)	211 (1)	195 (7)	194 (23)	270 (55)	240[a]	239 (100)	194 (13)		
23	8-Tetradecenyl	240[a]	194 (6)	241 (1)	239 (1)	211 (1)	195 (7)	194 (20)						
30	6-Pentadecenyl	254[a]	208 (7)	255 (3)	253 (1)	225 (1)	209 (9)	208 (24)	284 (27)	254 (6)	253 (34)	208 (15)	141 (26)	
31	8-Pentadecenyl	254[a]	208 (7)	255 (3)	253 (2)	225[a]	209 (9)	208 (23)						
36[b]	7-Hexadecenyl 8-Hexadecenyl	268[a]	222[a]	269 (5)	267 (3)	239[a]	223 (8)	222 (23)						
45	8-Heptadecenyl	282[a]	236 (6)	283 (3)	281 (2)	253[a]	237 (10)	236 (30)	312 (75)	282 (20)	281 (100)	236 (30)	141 (21)	171 (13)
53[b]	9-Octadecenyl 10-Octadecenyl	296[a]	250 (6)											
63	10-Nonadecenyl	310[a]	264[a]	311[a]	309 (2)	281 (7)	265 (12)	264 (36)	340 (87)	310 (23)	309 (100)	264 (21)	141 (8)	199 (12)
70[b,c]	10-Icosenyl 11-Icosenyl			325[a]	323 (3)	295 (1)	279 (7)	278[a]						
78	12-Henicosenyl	338[a]	292[a]	339 (7)	337 (3)	309 (2)	293 (12)	292 (45)	368 (66)	338 (15)	337 (60)	292 (16)	141 (80)	227 (15)
82[c]	13-Docosenyl			353 (10)	351 (3)	323 (5)	307 (15)	306 (48)						
86	14-Tricosenyl	366[a]	320 (3)	367 (10)	365 (3)	337 (4)	321 (12)	320 (60)						
89[d]	(15)-Tetracosenyl[c]	380[a]	334 (4)	381 (3)	379 (3)	351 (6)	335 (20)	334 (38)						
91[d]	(16)-Pentacosenyl[c]	394[a]	348 (3)	395 (11)	393 (3)	365 (6)	349 (16)	348 (65)						

[a] Not detected.
[b] Coeluting names are given in the order in which their DMDS derivatives are eluted.
[c] Not observed in the EI total ion gas chromatogram.
[d] Not observed in EI total ion chromatogram of DMDS derivatives; double bond position uncertain.

molecular mass of 254. The pair of peaks at *m/z* 70 and 71, possibly formed by the loss of, respectively, CHO and CO from the ion at *m/z* 99, are typical for δ-lactones of this type. Final confirmation that this component is hexadecan-5-olide was furnished by retention-time comparison with an authentic synthetic sample of the compound.

The preorbital secretion of the grysbok contains two constituents, 57 and 73, each having ions at *m/z* 48, 61, 75, and 103 in the lower mass range and two ions of high abundance differing by 15 mass units in the high mass range of their EI mass spectra. Information afforded by the CI(CH_4) mass spectra of the two compounds indicates that the ions at *m/z* 286 and 314 in their EI mass spectra are their respective molecular ions. Properties such as the molecular mass of the compounds, the facile loss of a methyl radical, the stability of the molecular ions, etc., suggest these compounds to be alkyl methyl sulfides. Taking component 57 as an example, the formation of some of the sulfur-containing ions in its spectrum can be formulated as shown in Scheme 1.

Components 57 and 73 were identified as heptadecyl methyl and methyl nonadecyl sulfides, respectively. It is interesting that reasonably abundant M^+, $(M-H)^+$, $(M-2H)^+$, $(M-3H)^+$, and $(M-SCH_3)^+$ ions are present in the CI(CH_4) spectra of both compounds. The $(M-SCH_3)^+$ ions, present in these mass spectra, are not observed in their EI spectra, possibly because the resulting primary carbocations undergo rapid fragmentation under EI conditions.

In certain respects the EI mass spectra of components 75 and 84 are similar to those of several hydroxyesters present in the dorsal secretion of the springbok, *Antidorcas marsupialis* (Le Roux, 1980; Burger et al., 1981b), and the interdigital secretion of the bontebok, *Damaliscus dorcas dorcas* (Nell, 1992). The hypothesis that these two components could possibly be similar hydroxyesters is supported by their EI and CI(CH_4) mass spectra. The high-resolution EI spectra of the hydroxyesters found in the dorsal secretion of the springbok (Le Roux, 1980) provided evidence supporting the formation of some of the diagnostically significant ions in the mass spectrum of, for example, 2-hydroxyicosan-1-yl acetate 84 (Figure 2) as shown in Scheme 2.

It is clear that the hydroxy group cannot be present in either the carboxyl function of the molecule or on the α-carbon atom of the alcohol residue of the molecule. Its presence on the β-carbon atom is confirmed by the ion at *m/z* 103. The formation of the prominent ion at *m/z* 74 in its mass spectrum can be rationalized in terms of the expulsion of a molecule of formaldehyde, followed by a McLafferty rearrangement (Scheme 3).

It can be accepted that the ions at *m/z* 357 and 355 in the CI(CH_4) mass spectrum of this compound (Figure 3) are generated by M+H and M−H processes, respectively, and that the ions at *m/z* 297 and 295 are formed by the expulsion of acetic acid from these respective ions. The unbranched structure

TABLE 5. DIAGNOSTIC IONS IN EI, CI(CH_4), AND CI(NO) MASS SPECTRA

No. in Figure 1	Alkadienol	EI		CI(CH_4)			
		M^+	$(M-18)^+$	$(M+1)^+$	M^+	$(M-1)^+$	$(M-17)^+$
9	4,7-Tridecadienol	196[a]	178 (0.5)	197 (25)	196 (4)	195 (13)	179 (23)
18	5,8-Tetradecadienol	210[a]	192 (0.5)	211 (20)	210 (10)	209 (15)	193 (25)
25	6,9-Pentadecadienol	224 (0.4)	206 (0.5)	225 (30)	224 (17)	223 (23)	207 (30)
40	8,10-Heptadecadienol	252 (0.4)	234 (0.2)	253 (31)	252 (28)	251 (56)	235 (31)

[a]Not detected.

of the alcohol residue of the molecule was confirmed by coinjection of an extract of the secretion with a synthetic sample of 2-hydroxyicosan-1-yl acetate synthesized in an Al_2O_3-catalyzed reaction of 1,2-epoxyicosane and acetic acid (Greyling, 1992). Component 75 was identified along similar lines as 2-hydroxyoctadecan-1-yl acetate.

Six γ-lactones, a δ-lactone, and the two hydroxyesters are the only chiral compounds identified in the preorbital secretion of the grysbok. The structure of the preorbital cavity is not so favorable for the survival of bacteria as that of the interdigital cavity of the bontebok and blesbok, but several bacteria, e.g., *Escherichia coli*, have been identified in the preorbital secretion of the grysbok (Ruppel, 1984). Lactones were found to be produced by the bacteria in the interdigital cavity in the bontebok and blesbok (Nell, 1992), and it is therefore possible that similar processes could be responsible for the presence of some of the constituents of the preorbital secretion of the grysbok. Regardless of whether these chiral constituents are synthesized in the gland or by bacteria, they are expected to be optically active. The determination of the absolute configuration of these compounds was not undertaken in the present study, however.

The double bonds in all of the unsaturated constituents of the secretion were assumed to possess *Z* configuration. In a previous study (Le Roux, 1980) representative examples of the various unsaturated compound types were isolated in quantities large enough to allow the recording of their ^{13}C NMR spectra. The presence in these spectra of a resonance at ca. 27.2 ppm, which can be ascribed to the allylic carbon atoms, was taken as unambiguous evidence that the double bonds in these compounds have the *Z* configuration, as the resonance of allylic carbon atoms in compounds with *E* configuration appears at ca. 32.5 ppm (Breit-

OF ALKADIENOLS PRESENT IN PREORBITAL SECRETION OF GRYSBOK

Diagnostic ion (%, normalized abundance)

CI(NO)							
$(M - 19)^+$	$(M + 30)^+$	$(M + 28)^+$	M^+	$(M - 1)^+$	$(M - 2)^+$	$(M - 18)^+$	$(M - 19)^+$
177 (31)	226[a]	224[a]	196 (23)	195 (91)	194[a]	178 (65)	177[a]
191 (30)	240 (45)	238[a]	210 (80)	209 (100)	208 (52)	192 (25)	191 (12)
205 (18)	254 (19)	252 (13)	224 (50)	223 (100)	222 (33)	206 (7)	205 (23)
233 (23)	282 (35)	280 (10)	252 (75)	251 (84)	250 (6)	234[a]	233[a]

maier et al., 1979; Wenkert et al., 1976). Careful comparison of retention times and of retention time increments confirmed this assumption. In this regard it is noteworthy that in the chemical characterization of the preorbital secretion of the oribi, *Ourebia ourebi*, in which more reference compounds were available, all the unsaturated constituents were found to possess the *Z* configuration (Mo et al., 1995). Both the oribi and the grysbok belong to the tribe Neotragini.

The compounds identified in the preorbital secretion of the grysbok are listed in Table 9, together with compounds identified in the previous study (Le Roux, 1980; Burger et al., 1981a) and information on the analytical methods employed.

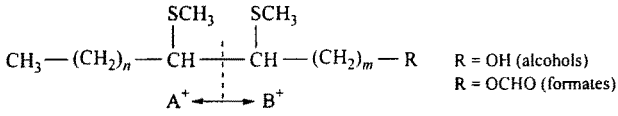
Unfortunately, a number of compounds still remained unidentified, mainly due to the lack of structurally significant information in their mass spectra and to the poor quality of the CI total ion current chromatogram. As the whole secretion was subjected to DMDS derivatization for the determination of the position of double bonds in the unsaturated constituents, the DMDS derivatives of the minor constituents could in some cases not be found in the total ion current chromatogram of the derivatized material, probably because the derivatives coeluted with certain constituents of the secretion. The unidentified constituents are also listed in Table 9 with mass spectral data, including the molecular ion or the ion at highest mass in a mass spectrum. The minor constituents, formic acid, hexanal, 2-heptanone, 2-octanone, 2-nonanone, and nonanoic acid, identified in the previous study of the preorbital secretion of the grysbok, are not visible in the total ion chromatogram depicted in Figure 1 and are not included in Table 9. In the previous study the coeluting alcohols (Z)-6-tridecen-1-ol and (Z)-7-tridecen-1-ol were incorrectly identified as (Z)-4-tridecen-1-ol.

TABLE 6. DIAGNOSTIC IONS IN EI, CI(CH_4), AND CI(NO) MASS SPECTRA OF ALKADIENYL FORMATES PRESENT IN PREORBITAL SECRETION OF GRYSBOK

No. in Figure 1	Formate	Diagnostic ion (%, normalized abundance)									
		EI		CI(CH_4)				CI(NO)			
		M^+	$(M-46)^+$	$(M+1)^+$	M^+	$(M-1)^+$	$(M-45)^+$	$(M+30)^+$	$(M+1)^+$	M^+	$(M-1)^+$
21	(5,8)-Tetradecadienyl[a]	238[b]	192[b]	239[b]	238 (9)	237 (38)	193 (47)	268 (22)	239[b]	238 (100)	237[b]
29	6,9-Pentadecadienyl	252 (3)	206 (0.5)	253 (15)	252 (25)	251 (30)	207 (60)	282 (48)	253 (20)	252 (100)	251 (40)
44	8,11-Heptadecadienyl	280 (3)	234[b]	281 (7)	280 (48)	279 (35)	235 (35)	310 (60)	281 (20)	280 (100)	279 (42)
62	(10,13)-Nonadecadienyl[a]	308 (3)	262[b]	309 (20)	308 (25)	307 (17)	263 (23)	338 (33)	309 (60)	308 (100)	307 (17)
77	(12,15)-Henicosadienyl[a]	336 (3)	290[b]	337[b]	336 (1)	335 (6)	291 (4)	366 (41)	337 (11)	336 (56)	335[b]

[a] Not located in the EI total ion chromatogram of the DMDS derivatives; double bond position uncertain.
[b] Not detected.

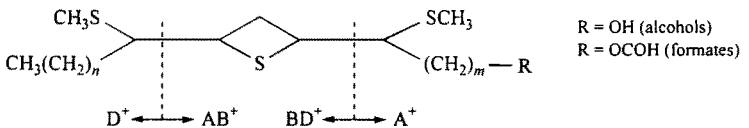
TABLE 7. DIAGNOSTIC IONS IN EI MASS SPECTRA OF DMDS DERIVATIVES OF MONOUNSATURATED ALCOHOLS AND FORMATES PRESENT IN PREORBITAL SECRETION OF GRYSBOK



No. in Figure 1	Alkenol/alkenyl formate	Diagnostic ion (%, normalized abundance)			
		M^+	$(M-47)^+$	A^+	B^+
4	6-Dodecen-1-ol	278 (70)	231[a]	131 (100)	147 (47)
10[b]	6-Tridecen-1-ol	292 (57)	245[a]	145 (100)	147 (46)
	7-Tridecen-1-ol	292 (70)	245[a]	131 (66)	161 (67)
19[b]	8-Tetradecen-1-ol	306 (77)	259 (3)	145 (100)	161 (66)
	7-Tetradecen-1-ol	306 (49)	259 (7)	131 (99)	175 (100)
26[b]	8-Pentadecen-1-ol	320 (48)	273 (8)	145 (100)	175 (95)
	6-Pentadecen-1-ol	320 (28)	273 (3)	173 (100)	147 (45)
34[b]	8-Hexadecen-1-ol	334 (44)	287 (9)	159 (100)	175 (70)
	7-Hexadecen-1-ol	334 (60)	287 (15)	159 (100)	175 (70)
41	8-Heptadecen-1-ol	348 (23)	301 (5)	173 (100)	175 (72)
58	10-Nonadecen-1-ol	376 (20)	329 (6)	173 (100)	203 (97)
71	12-Henicosen-1-ol	404 (19)	357 (5)	173 (85)	231 (100)
88	14-Tricosen-1-ol	432 (19)	385[a]	173 (65)	259 (100)
16	7-Tridecenyl formate	320 (24)	273[a]	131 (100)	189 (45)
22	7-Tetradecenyl formate	334 (33)	287[a]	145 (100)	189 (65)
23	8-Tetradecenyl formate	334 (30)	287 (7)	131 (85)	203 (100)
30	6-Pentadecenyl formate	348 (13)	301 (3)	173 (85)	175 (14)
31	8-Pentadecenyl formate	348 (33)	301 (8)	145 (100)	203 (95)
36	7-Hexadecenyl formate	362 (40)	315 (17)	173 (100)	189 (40)
	8-Hexadecenyl formate	362 (29)	315 (10)	159 (100)	203 (98)
45	8-Heptadecenyl formate	376 (17)	329 (5)	173 (100)	203 (90)
53	9-Octadecenyl formate	390 (23)	343 (5)	173 (79)	217 (100)
	10-Octadecenyl formate	390 (30)	343 (8)	159 (33)	231 (65)
63	10-Nonadecenyl formate	404 (12)	257 (3)	173 (60)	231 (100)
70[c]	10-Icosenyl formate	418 (17)	371 (10)	173 (38)	245 (100)
	11-Icosenyl formate	418 (32)	317 (22)	187 (68)	231 (100)
78	12-Henicosenyl formate	432 (7)	385 (2)	173 (24)	259 (100)
82[c]	13-Docosenyl formate	446 (13)	399 (3)	173 (35)	273 (100)
86	14-Tricosenyl formate	460 (6)	413 (3)	173 (39)	287 (100)

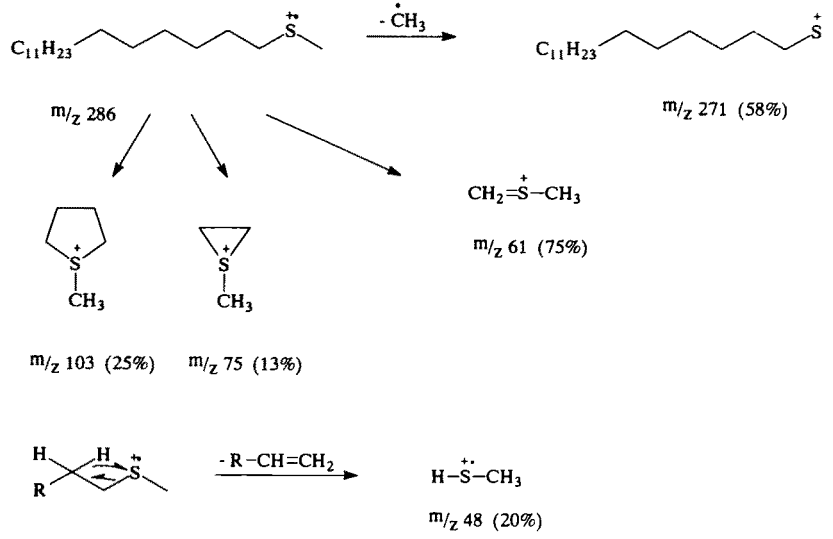
[a] Not detected.
[b] Coeluting isomers given in the order in which their DMDS derivatives are eluted.
[c] Not located in EI and CI total ion chromatograms.

TABLE 8. DIAGNOSTIC IONS IN EI MASS SPECTRA OF DMDS DERIVATIVES OF DIUNSATURATED ALCOHOLS AND FORMATES PRESENT IN PREORBITAL SECRETION OF GRYSBOK

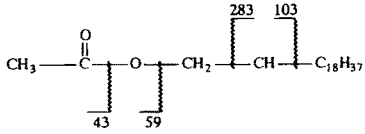


No. in Figure 1	Alkadienol/alkadienyl formate	Diagnostic ion (%, normalized abundance)							
		M^+	$(M-47)^+$	$(M-95)^+$	BD^+	D^+	AB^+	A^+	$(BD-48)^+$
9	4,7-Tridecadien-1-ol	322 (70)	275[a]	227[a]	203 (32)	131 (47)	191 (32)	119[a]	155 (42)
25	6,9-Pentadecadien-1-ol	350 (90)	303 (35)	255 (32)	203 (31)	131 (62)	219 (4)	147 (19)	155 (100)
40	8,11-Heptadecadien-1-ol	378 (80)	331[a]	283 (43)	203 (25)	131 (64)	247[a]	175 (4)	155 (57)
29	6,9-Pentadecadienyl formate	378 (65)	331 (35)	283 (32)	203 (31)	131 (79)	247 (4)	175 (7)	155 (100)
44	8,11-Heptadecadienyl formate	406 (18)	359 (27)	311 (27)	203 (26)	131 (22)	275[a]	203 (61)	155 (100)

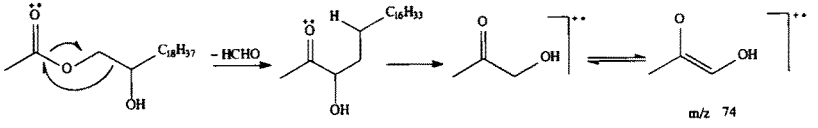
[a] Not detected.



SCHEME 1.



SCHEME 2.



SCHEME 3.

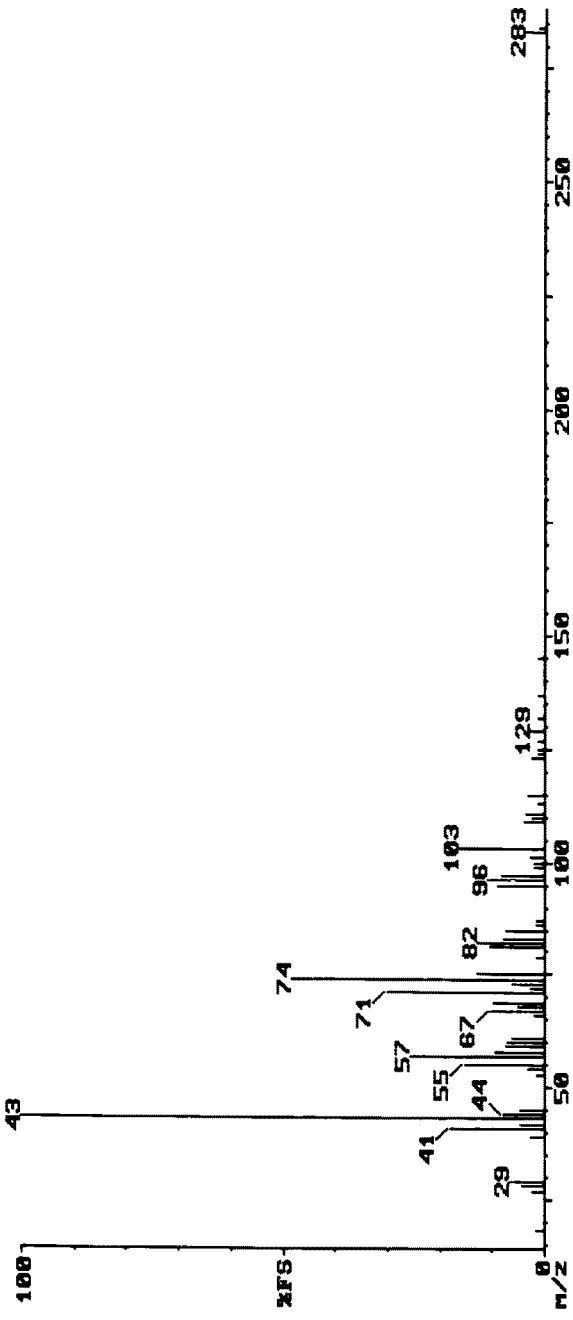


FIG. 2. EI mass spectrum of constituent 84 (2-hydroxyicosan-1-yl acetate).

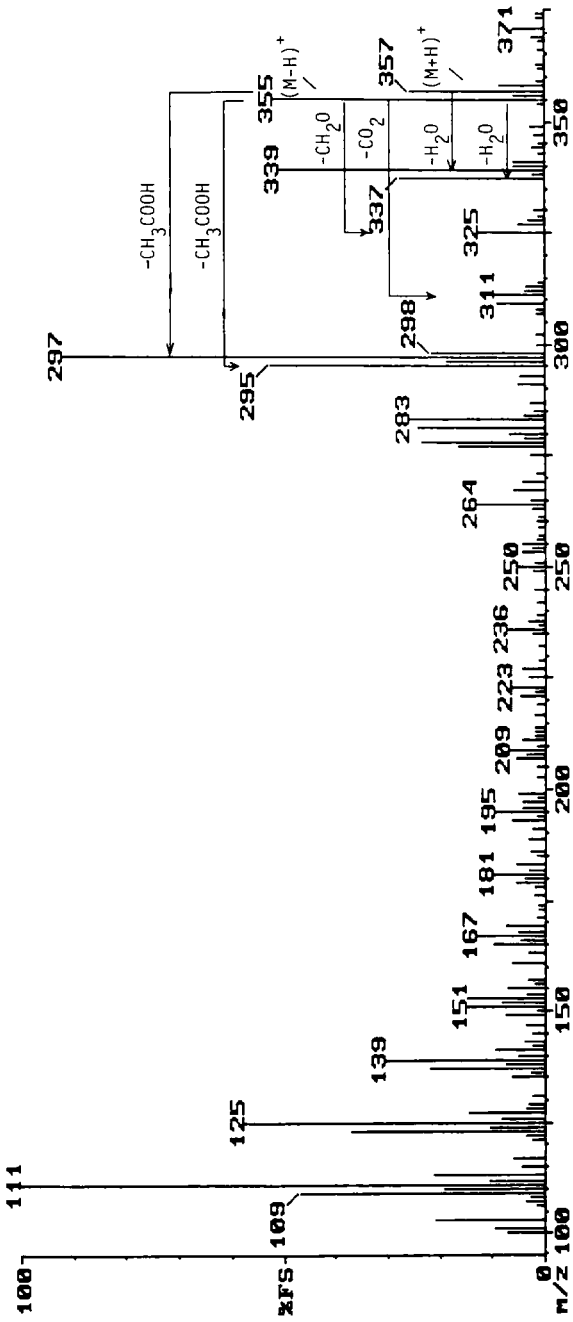


FIG. 3. CI(CH_4) mass spectrum of constituent 84 (2-hydroxyicosan-1-yl acetate).

TABLE 9. CONSTITUENTS OF PREORBITAL SECRETION OF GRYSBOK

No. in Figure 1	Compounds	Quantity (pg/animal)	Analytical methods
1	Undecan-1-ol	30	a,b,c,d,i
5	Dodecan-1-ol	400	a,b,c,d,f,g,i
11	Tridecan-1-ol	1310	a,b,c,d,f,g,i
20	Tetradecan-1-ol	775	a,b,c,d,f,g,i
28	Pentadecan-1-ol	570	a,b,c,d,f,g,i
3	Undec-1-yl formate	7	b,c,i
8	Dodec-1-yl formate	40	b,c,d,i
17	Tridec-1-yl formate	140	b,c,d,i
24	Tetradec-1-yl formate	150	a,b,c,d,f,g,i
33	Pentadec-1-yl formate	370	a,b,c,d,f,i
38	Hexadec-1-yl formate	20	a,b,c,d,f,g
49	Heptadec-1-yl formate	75	b,c,d,i
56	Octadec-1-yl formate	95	a,b,c,d,f
66	Nonadec-1-yl formate	740	a,b,c,d,f,g
72	Icos-1-yl formate	340	a,b,c,d,f,g,i
80	Henicos-1-yl formate	1220	a,b,c,d,f,g,h,i
83	Docos-1-yl formate	565	a,b,c,f,g,i
87	Tricos-1-yl formate	4110	a,b,c,f,g,h
90	Tetracos-1-yl formate	1555	a,b,c,f,i
92	Pentacos-1-yl formate	6108	a,b,c,f
4	(Z)-6-Dodecen-1-ol	10	b,c,e
10	(Z)-6-Tridecen-1-ol	270	b,c,d,e
	(Z)-7-Tridecen-1-ol		b,c,d,e
19	(Z)-8-Tetradecen-1-ol	445	b,c,d,e
	(Z)-7-Tetradecen-1-ol		b,e
26	(Z)-8-Pentadecen-1-ol	1260	b,c,d,e
	(Z)-6-Pentadecen-1-ol		a,b,c,e,f,g,h
34	(Z)-8-Hexadecen-1-ol	50	b,e
	(Z)-7-Hexadecen-1-ol		b,e
41	(Z)-8-Heptadecen-1-ol	435	a,c,e,f,g,h
58	(Z)-10-Nonadecen-1-ol	50	a,c,f
71	(Z)-12-Henicosen-1-ol	25	b,e
88	(Z)-14-Tricosen-1-ol	30	b,e
16	(Z)-7-Tridecen-1-yl formate	50	b,e
22	(Z)-7-Tetradecen-1-yl formate	15	b,c,e
23	(Z)-8-Tetradecen-1-yl formate	20	b,c,e
30	(Z)-6-Pentadecen-1-yl formate	195	b,c,d,e
31	(Z)-8-Pentadecen-1-yl formate	50	b,c,e
36	(Z)-7-Hexadecen-1-yl formate	10	b,c,e
	(Z)-8-Hexadecen-1-yl formate		b,e
45	(Z)-8-Heptadecen-1-yl formate	715	a,b,c,d,e,f,g,h
53	(Z)-9-Octadecen-1-yl formate	10	b,c,e
	(Z)-10-Octadecen-1-yl formate		b,e
63	(Z)-10-Nonadecen-1-yl formate	780	a,b,c,d,e,f,g
70	(Z)-10-Icosen-1-yl formate	10	b,e
	(Z)-11-Icosen-1-yl formate		b,e

TABLE 9. Continued

No. in Figure 1	Compounds	Quantity (pg/animal)	Analytical methods
78	(Z)-12-Henicosen-1-yl formate	665	a,b,c,d,e,f,g
82	(Z)-13-Docosen-1-yl formate	20	b,e
86	(Z)-14-Tricosen-1-yl formate	1505	a,b,c,e,f,g
89	(Z)-15-Tetracosen-1-yl formate	25	b,c,k
91	(Z)-16-Pentacosen-1-yl formate	1190	b,c,k
9	(4Z,7Z)-4,7-Tridecadien-1-ol	60	b,c,d,k
18	(5Z,8Z)-5,8-Tetradecadien-1-ol	140	b,c,d,k
25	(6Z,9Z)-6,9-Pentadecadien-1-ol	2645	a,b,c,d,e,f,g,h
40	(8Z,10Z)-8,10-Heptadecadien-1-ol	265	a,b,c,d,e,f,g,h
21	(5Z,8Z)-5,8-Tetradecadien-1-yl formate	10	b,c,d,j,k
29	(6Z,9Z)-6,9-Pentadecadien-1-yl formate	180	b,c,d,e
44	(8Z,11Z)-8,11-Heptadecadien-1-yl formate	150	b,c,d,e
62	(10Z,13Z)-10,13-Nonadecadien-1-yl formate	80	b,c,d,k
77	(12Z,15Z)-12,15-Henicosadien-1-yl formate	70	b,c,d,k
2	Dodecanal	15	b,c,d,i
7	Tridecanal	50	b,c,d
6	(Z)-3-Tridecenal	35	b,c,k
27	Tetradecanoic acid	trace	b,d,i
35	Pentadecanoic acid	trace	b,d,i
42	Hexadecanoic acid	655	a,b,c,d,f,i
50	Heptadecanoic acid	15	b,d
59	Octadecanoic acid	110	a,b,d,f,i
74	Icosanoic acid	60	b,d
43	Pentadecan-4-olide	50	b
52	Hexadecan-4-olide	120	b,c,d,i
61	Heptadecan-4-olide	20	b,c,d
69	Octadecan-4-olide	5	b,c,i
81	Icosan-4-olide	25	b,i
54	Hexadecan-5-olide	20	b,c,d,i
57	Heptadec-1-yl methyl sulfide	80	b,c,i
73	Methyl nonadec-1-yl sulfide	30	b,c
75	2-Hydroxyoctadecan-1-yl acetate	15	b,c
84	2-Hydroxyicosan-1-yl acetate	215	b,c,i
12	Unsaturated aldehyde	*m/z* 81 (100), 41 (35), 67 (28), 83 (25), and 194 (1)	
13	Unidentified	*m/z* 79 (100), 41 (70), 67 (68), 91 (65), 55 (60), and 192 (1)	
14	Unidentified	*m/z* 81 (100), 79 (58), 41 (40), 67 (37), 91 (32), 77 (30), and 192 (2)	
15	Unidentified	*m/z* 41 (100), 55 (100), 83 (78), 57 (68), 67 (67), 84 (66), 43 (65), and 196 (3)	
32	Unidentified	*m/z* 55 (100), 41 (83), 57 (70), 67 (66), 69 (55), 68 (54), 96 (53), and 208 (5)	

TABLE 9. Continued

No. in Figure 1	Compounds	Quantity (pg/animal)	Analytical methods
37	Unidentified	*m/z* 55 (100), 82 (88), 41 (86), 69 (70), 57 (69), 67 (60), 96 (54), and 222 (6)	
39	Alkyl phthalate (contaminant)		
40	Unidentified	*m/z* 67 (100), 81 (68), 55 (57), 41 (48), 79 (45), 54 (42), 95 (39), and 252 (0.5)	
46	Unidentified	*m/z* 43 (100), 55 (93), 41 (88), 57 (75), 69 (70), 83 (62), 66 (51), and 281 (2)	
47	Unidentified	*m/z* 55 (100), 41 (85), 67 (61), 69 (53), 81 (51), 98 (38), 82 (37), and 251 (2)	
48	Impure peak		
51	Unidentified	*m/z* 55 (100), 82 (85), 41 (80), 67 (78), 81 (69), 96 (60), 80 (56), and 236 (4)	
55	Unidentified	*m/z* 67 (100), 55 (98), 41 (82), 82 (72), 81 (70), 68 (60), 69 (51), 95 (48), and 280 (2)	
60	Unidentified	*m/z* 55 (100), 82 (83), 41 (69), 96 (63), 67 (55), 69 (53), 43 (49), 57 (48), and 250 (8)	
64	Unidentified	*m/z* 55 (100), 41 (68), 67 (66), 69 (64), 81 (53), 83 (48), 82 (45), 95 (41), and 262 (4)	
65	Unidentified	*m/z* 55 (100), 41 (88), 67 (78), 82 (68), 81 (67), 80 (66), 68 (54), 69 (50), and 280 (2)	
68	Unidentified	*m/z* 80 (100), 67 (73), 55 (68), 41 (58), 81 (57), 68 (44), 94 (43), 95 (37), and 262 (10)	
76	Unidentified	*m/z* 55 (100), 41 (82), 67 (70), 81 (53), 68 (47), 95 (46), 82 (38), 43 (36), and 306 (14)	
79	Unidentified	*m/z* 55 (100), 41 (78), 80 (77), 67 (74), 81 (70), 82 (58), 68 (50), 67 (49), 95 (47), and 308 (3)	
85	Unidentified	*m/z* 43 (100), 57 (66), 55 (60), 83 (56), 69 (54), 97 (48), 41 (38), 82 (35), 71 (33), and 356 (0.3)	

a: Identified in previous study; b: low-resolution mass spectrum; c: CI(CH_4) mass spectrum; d: CI(NO) mass spectrum; e: EI mass spectrum of dimethyl disulfide derivative; f: high-resolution mass spectrum; g: ^1H NMR spectrum; h: ^{13}C NMR spectrum; i: retention time comparison; j: detected using a polar column; k: double bond position(s) uncertain.

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PRESERVATION OF SALICACEAE LEAVES FOR PHYTOCHEMICAL ANALYSES: FURTHER ASSESSMENT

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Abstract—The chemistry of the plant family Salicaceae has been of interest to researchers as diverse as chemical ecologists, chemosystematists, and paper chemists. Continuing the debate on proper methods for preservation of plant material prior to analysis, vacuum-drying was recently advocated, because freeze-drying may cause degradation of phenolic glycosides. This study was conducted to clarify the consequences of freeze-drying for foliar secondary chemicals and to evaluate the consequences of vacuum-drying for primary compounds (protein and carbohydrates). Leaves of quaking aspen (*Populus tremuloides*) were flash-frozen in liquid nitrogen and freeze-dried or vacuum-dried at room temperature. We then analyzed samples for levels of salicortin and tremulacin (phenolic glycosides), condensed tannins, nitrogen, soluble protein, sugars, and starch. Freeze-drying did not alter the concentrations of phenolic glycosides or tannins, relative to vacuum-drying. Freeze-drying did cause a small and inexplicable decline in nitrogen and soluble protein. Vacuum-drying, however, reduced starch concentrations by 38%. We suggest that the vacuum-drying method be used in studies in which carbohydrates are of no interest. For studies measuring carbohydrates, however, freeze-drying is a better alternative, and should effect no changes in levels of secondary compounds if samples are not allowed to thaw during the drying process.

Key Words—Carbohydrates, leaf preservation, phenolic glycosides, *Populus*, protein, Salicaceae, salicortin, tannins, tremulacin.

INTRODUCTION

Members of the plant family Salicaceae have played prominent roles in research on chemosystematics (Steele and Ronald, 1973; Julkunen-Tiitto, 1986) and

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chemical ecology, including interactions with insects (Lindroth et al., 1988; Bryant et al., 1987), mammals (Basey et al., 1990; Reichardt et al., 1990), birds (Jakubas, 1989), fungi (Kruger and Manion, 1994), and multiple trophic levels (Pasteels et al., 1983; Smiley et al., 1985). These studies have emphasized secondary compounds, most notably condensed tannins and a variety of phenolic glycosides (Palo, 1984; Julkunen-Tiitto, 1986; Bryant et al., 1987; Lindroth et al., 1987a). Moreover, because the secondary chemistry of members of the Salicaceae exhibits substantial phenotypic and genotypic variation, these species have been the subject of studies on induced chemical defenses (Clausen et al., 1989), resource availability (Larsson et al., 1986; Bryant et al., 1987) and genotype by environment interactions (Price et al., 1989; Julkunen-Tiitto et al., 1993).

Given the importance of secondary compounds of the Salicaceae for understanding diverse ecological and evolutionary interactions, proper methods for preservation of foliar material prior to chemical analysis are of utmost importance. They have also been the subject of debate. Low-temperature oven-drying was formerly widely used for processing of leaf and bark tissues. Lindroth and Pajutee (1987) documented hydrolytic degradation and interconversion of phenolic glycosides in aspen (*Populus tremuloides*) samples subjected to oven-drying or slow freezing (−20°C) followed by freeze-drying, and advocated processing of fresh leaf material. We later found that flash-freezing of leaf tissue followed by freeze-drying did not alter phenolic glycoside concentrations (Lindroth et al., 1993) and adopted this method for large-scale studies that precluded collection and analysis of leaf tissues on the same day. Julkunen-Tiitto and Tahvanainen (1989) showed that air-drying of *Salix* tissues effected no qualitative changes and only minor quantitative changes in phenolic glycoside compositions, but did not measure responses of tannins. Most recently, Orians (1995) reported that freeze-drying and air-drying reduced levels of phenolic glycosides and condensed tannins, respectively, in *Salix* leaves. He suggested vacuum-drying of fresh leaves as the method of choice for researchers interested in concentrations of both types of secondary compounds.

Vacuum-drying of fresh material provides obvious, practical advantages over previous methods that relied on flash-freezing of tissues in liquid nitrogen prior to freeze-drying, but this new approach also raises some questions: (1) Are results from earlier work on freeze-dried tissues valid? (2) Does vacuum-drying without flash-freezing cause changes in levels of other plant chemicals routinely measured in phytochemically oriented studies? Here we report that when properly executed, flash-freezing followed by freeze-drying does not alter levels of phenolic glycosides or tannins. This method also helps to preserve concentrations of carbohydrates, although levels of protein may be marginally affected.

METHODS AND MATERIALS

Leaf Material. Leaves were collected from a single aspen tree (containing intermediate levels of phenolic glycosides and condensed tannins) in the University of Wisconsin Arboretum in early September 1994. Approximately 70 g of fully expanded, mature leaves were clipped at the petiole and transported to the laboratory on ice. Leaves were then mixed together and divided into twelve 5-g aliquots. For the vacuum-drying treatment, six aliquots were placed directly into a Virtis freeze-dryer and dried at room temperature for four days. For the freeze-drying treatment, six aliquots were flash-frozen in liquid nitrogen, then placed into a precooled (−25°C) drying chamber of another Virtis freeze-dryer, and dried at low temperature for four days. Following drying, leaves were ground (40-mesh) and stored at −20°C until analyzed.

Chemical Analyses. Leaf material was analyzed for the major carbon-based secondary compounds (phenolic glycosides and condensed tannins) as well as for primary compounds of recognized importance in plant–insect interactions. The latter included Kjeldahl nitrogen (as an index of total protein), soluble protein, hexoses, sucrose, and starch. With the exception of the nitrogen assay, all our chemical assays relied on solvent extraction; data presented represent the extractable portion occurring in aspen leaf tissue.

Quaking aspen produces a suite of four phenolic glycosides, of which salicortin and tremulacin constitute >90% by mass (Lindroth et al., 1987b). We measured concentrations of these two compounds by high-performance thin-layer chromatography (HPTLC). We extracted 25 mg of tissue in 1 ml methanol for 15 min, with sonication. Duplicate 1-μl aliquots of the extracts were then applied to HPTLC plates (Silica gel 60, 10 × 20 cm); purified salicortin and tremulacin standards were applied similarly. Plates were developed in 6:1:1 methylene chloride–methanol–tetrahydrofuran, then scanned (274 nm) in a Camag TLC Scanner II. Chromatograms were analyzed using Camag TLC evaluation software (CATS 3.11). Chromatograms were also visually inspected for the presence of salicin, which typically occurs in aspen tissues near the limit of detection with our standard HPTLC technique.

We measured condensed tannin concentrations by a modification of the method of Porter et al. (1986). Leaf material (50 mg) was extracted (4 × 1 ml, 4°C, 30 min with sonication) with 70% acetone (containing 10 mM ascorbic acid as antioxidant). For hydrolysis, duplicate samples were immersed in boiling water for 50 min. Condensed tannins purified from aspen leaves (method of Hagerman and Butler, 1980) served as a standard.

For Kjeldahl nitrogen determinations, 25-mg quantities of leaf tissue were digested according to the procedure of Parkinson and Allen (1975). We quantified nitrogen contents of the digests in duplicate samples by a micro-Nessler-

ization technique (Lang, 1958), using glycine–*p*-toluene–sulfonic acid (5.665% N) as a standard. We also measured soluble protein, using a modification of the procedure of Jones et al. (1989). Samples of 25 mg were extracted in 0.1 N NaOH (containing 0.05% sodium dodecyl sulfate to prevent precipitation of protein). Extracts were analyzed in duplicate and bovine serum albumin served as the protein standard.

Carbohydrate concentrations were measured by the method of Schoeneberger et al. (1995). This procedure provides for separate quantification of hexoses (glucose and fructose), sucrose, and starch in 25-mg samples. Fructose, sucrose, and starch are enzymatically converted to glucose, which is then quantified indirectly via reduction of NADP to NADPH.

RESULTS AND DISCUSSION

Concentrations of secondary compounds in aspen leaves were not affected by the method of leaf preservation (Figure 1). Our results for phenolic glycosides differ from those of Orians (1995), who found that freeze-drying reduced concentrations of salicortin by 45–73% in *Salix* leaves. Orians used a freeze-dryer without temperature control of the drying chamber and acknowledged that some thawing of leaf tissue after flash-freezing may have contributed to the loss of salicortin (and 2′-cinnamoyl salicortin). Moreover, Orians detected substantial increases in levels of salicin in freeze-dried samples, whereas we detected none. Salicin is formed upon hydrolytic degradation of salicortin and tremulacin, as is likely to occur during thawing of frozen leaf tissue. Thus the reductions in phenolic glycoside concentrations reported by Orians were likely due to thawing of leaf tissue, not freeze-drying per se. These results emphasize the importance of maintaining samples in the frozen state when freeze-drying is used for sample preservation. Our results for condensed tannins agree with those reported by Orians (1995), who also found that freeze-drying did not alter concentrations.

To our surprise, freeze-drying altered foliar protein levels, although only minimally. Concentrations of total nitrogen and soluble protein declined by 7 and 10%, respectively, in freeze-dried tissues. Because flash-freezing and freeze-drying should be benign with respect to protein concentrations, the reasons for the apparent decline are unknown.

Carbohydrate concentrations are likely to be susceptible to leaf preservation methods, as starch may be labile under conditions that fail to inhibit activity of the starch-degrading enzyme α-amylase. Such appears to be the case for vacuum-dried leaves. Although overall starch levels were low in our late-season leaves, concentrations declined 38% in vacuum- versus freeze-dried samples. As expected, the reduction in percent leaf dry mass for starch was reflected by a corresponding increase in percent leaf dry mass for hexoses (glucose), although

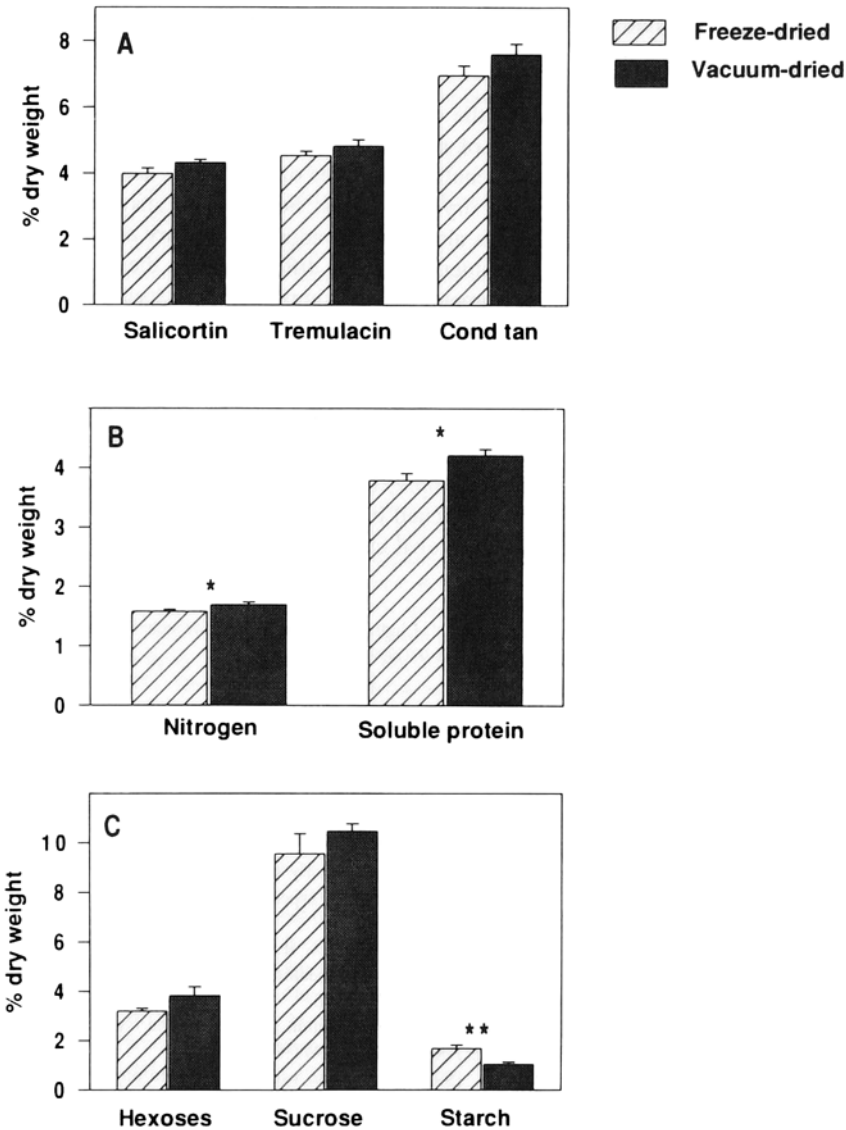

FIG. 1. Effects of preservation method on phytochemical composition of quaking aspen leaves: (A) Secondary compounds (cond tan = condensed tannins); (B) nitrogen and protein; (C) carbohydrates. Vertical lines indicate 1 SE. Means compared by t tests (*$0.01 < P < 0.05$; **$0.001 < P < 0.01$). P values for all nonsignificant comparisons were > 0.10.

the increase was not statistically significant. Our results suggest that freeze-drying is preferable to vacuum-drying for studies in which leaf carbohydrates are of interest.

In conclusion, the answer to the question of whether results from earlier work on the secondary chemistry of aspen are valid is "yes"; freeze-drying does not alter concentrations of phenolic glycosides or condensed tannins when samples remain frozen during the drying process. Second, the answer to the question of whether drying methods alter concentrations of primary compounds is also "yes", although the constituents affected differ between the two preservation methods tested. We recommend that the simple vacuum-drying method advocated by Orians (1995) be used for studies in which carbohydrate concentrations are not of interest. For example, because gypsy moth performance is related to aspen phenolic glycosides but not to carbohydrates (Hemming and Lindroth, 1995), our future studies will use the vacuum-drying method. We also recommend, however, that freeze-drying be used for studies in which leaf carbohydrates are of interest. For example, because starch concentrations may approach 10% leaf dry weight in enriched carbon dioxide environments (Lindroth et al., 1993, 1995), our future global change research will use the freeze-drying method.

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LEARNING AND SIBLING ODOR PREFERENCE IN JUVENILE ARCTIC CHAR, *Salvelinus alpinus* (L.)

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Abstract—The importance of learning for sibling odor preference in juvenile Arctic char was analyzed in the present study. Fish were reared in the following eight conditions: (1) communally with siblings for 15 months; (2) communally with siblings for 17 months; (3) in isolation since fertilization; (4) in isolation since fertilization and exposed to sibling scent during the whole rearing period; (5) in isolation since fertilization and exposed to sibling scent from time of free swimming; (6) in isolation since fertilization and exposed to sibling scent during the whole rearing period, except two months without scent until testing; (7) in isolation since fertilization and exposed to sibling scent from time of free swimming, except two months without scent until testing; and (8) communally with siblings followed by a two-month isolation until testing. Char were followed individually in a Y-maze (fluviarium test) with a video-computer-based image analysis system for 12 hr. Sibling-scented water was supplied to one lateral half of the test area and water from nonsiblings on the opposite half. Isolated individuals without any preexposure to siblings showed no significant preference. Test fish reared with siblings and those that had been reared in isolation but exposed to sibling scent until testing preferred water conditioned by their own siblings. Isolated fish that had been exposed to sibling scent since fertilization, or since free swimming, followed by a two-month period with only pure water, showed no significant preference. Char isolated for two months after being communally reared preferred water scented by siblings. The results demonstrated that behavioral discrimination between siblings and nonsibling odors occurred after total isolation (isolated both from siblings and sibling odors) only in individuals that had been communally reared. This may suggest that social interactions are important for learning and long-term memory of sibling odors in Arctic char.

Key Words—Arctic char, attraction, fish, fluviarium, kinship, odor, salmonids, *Salvelinus alpinus*, siblings.

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INTRODUCTION

Behavioral discrimination of siblings or close relatives has been demonstrated in social organisms of various taxonomic groups (reviews: Holmes and Sherman, 1983; Blaustein and O'Hara, 1986; Fletcher and Michener, 1987; Waldman, 1987, 1988). Such behavior has been proposed to be part of a kin recognition or discrimination mechanism. It has been suggested that the ability to discriminate kin may increase an individual's inclusive fitness (its own and close relatives' reproductive success) and decrease the risk of inbreeding (Hamilton, 1964).

Four mechanisms have previously been proposed for kin recognition (Blaustein, 1983; Holmes and Sherman, 1983), (1) spatial distribution—relatives are distributed predictably in space; (2) familiarity or previous association, i.e., recall of features learned during previous social interactions with a special group of kin; (3) phenotypic matching—an individual learns the phenotypes of relatives or itself, and relationships with other individuals are determined by comparing the learned phenotype with that of an unfamiliar conspecific; and (4) both the phenotype and its recognition have genetic bases and no learning is involved. This division into four mechanisms has recently been revised by some authors. Waldman (1988, 1991) divided kin recognition mechanisms into two categories: indirect (item 1 above) and direct (items 2–4 above) recognition. He suggested that direct recognition is composed of a series of events. Individuals expose labels, which are perceived by a second individual, and due to the fit of these labels to a template (innate or acquired) recognition occurs, which, in an appropriate social context, may lead to behavioral discrimination. It is the behavioral response that tells the observer that recognition and discrimination has occurred.

In fish, very little is known about the ability to distinguish between individuals or groups with different degrees of relationship. Most studies have been concerned with the ability of salmonids to use olfaction for kinship discrimination (Olsén, 1992). In salmonids sibling discrimination by chemical cues has, up to now, been demonstrated in three species, i.e., coho salmon, *Oncorhynchus kisutch*, Arctic char, *Salvelinus alpinus*, and rainbow trout, *Oncorhychus mykiss* (Quinn and Busack, 1985; Quinn and Hara, 1986; Olsén, 1989; Winberg and Olsén, 1992; Brown et al., 1993). Test fishes preferred water scented by unfamiliar siblings over unfamiliar nonsiblings. These studies indicated that the olfactory cues acting as intraspecific attractants are genetically determined. The results also suggest that odor traits from siblings are learned and then used as templates against which unfamiliar traits are compared.

Winberg and Olsén (1992) demonstrated that individual Arctic char kept in isolation since fertilization showed no preference when given a choice between two water currents scented by siblings and nonsiblings. The results indicated that juvenile Arctic char either learn the odor quality of siblings but not their

own smell, or they do not use the information (i.e., no self-matching). The study also indicated that the olfactory features are learned at an early age, because fish that were placed with siblings for 50–62 days after 15 months in isolation still showed no preference. In the present study we try to analyze the impact of isolation and communal rearing for sibling odor preference in juvenile Arctic char. Isolated fish were exposed to sibling-scented water either from the time of fertilization or from free swimming until testing. In some other isolated individuals, sibling odors was removed for two months before testing. Moreover, char were placed in isolation for two months after they had been reared communally since fertilization. The results suggest that communal rearing is important for permanent learning of sibling odors in Arctic char.

METHODS AND MATERIALS

Fish. Two full-sibling families were created by using two pairs of Arctic char at the Kälarne hatchery of the Swedish National Board of Fisheries. The parents were offspring of individuals caught in Lake Hornavan, Lapland, Sweden. Egg lots from these two pairs, family A and family B, were transported to the Department of Limnology, Uppsala University, within 12 hr of fertilization at the hatchery.

Most eggs from family A (ca. 300) were placed together and supported for communal rearing (called rearing regimes 1a and 1b in Figure 1) and hereafter referred to as test groups 1a and 1b. These eggs from family A and the fertilized eggs from family B (ca. 300) (B fish were only used as odor donors in fluviarium tests, not as test fish) were placed in two separate troughs (41 cm long, 69 cm wide, and 12.5 cm deep) supplied with 800 ml/min of aerated tap water. These two families were later transferred to a fiberglass holding tank (500 liters), supplied with 1200 ml/min of aerated tap water.

Twenty-five fertilized eggs from family A were placed individually in a trough with separate compartments (58 × 58 × 45 mm deep). Each compartment had its own water supply and outlet and was supplied with 40 ml/min of aerated Uppsala tap water (oxygen at least 80% of saturation). Eleven of the fish were reared in total isolation (i.e., only supply of tap water) up to testing (rearing regime 2). When fish became free swimming (four months after fertilization), the supply of sibling-scented water was started to the remaining 14 (rearing regimes 4a and 4b). Fish supported to rearing regime 4a were exposed to sibling odors up to testing as group 4a. At 15–16 months after fertilization, the supply of sibling odors was stopped to seven fish, giving rearing regime 4b (group 4b), and the group was tested two months later (median 56 days; range 52–60).

Another 25 eggs from family A were individually placed in a similar trough

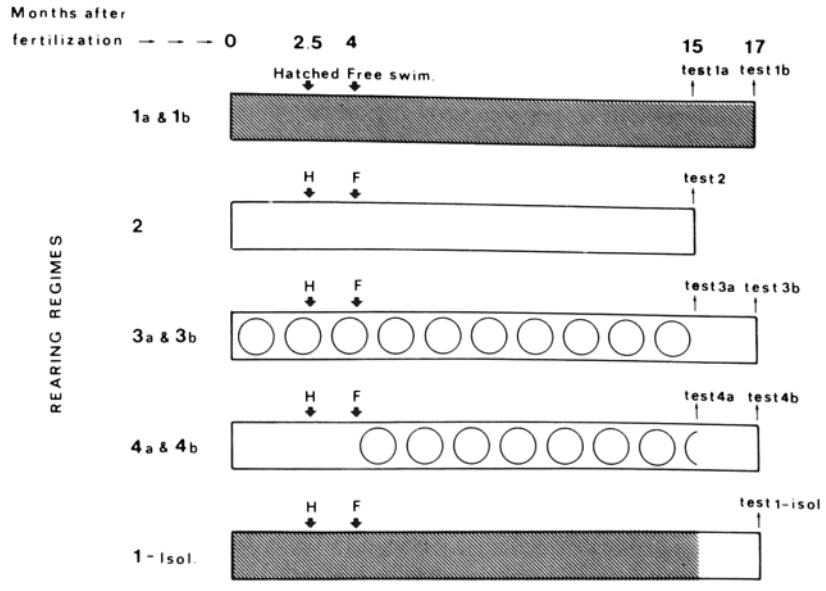

FIG. 1. Diagram of the different rearing regimes giving rise to eight different groups tested in the fluviarium. Two groups of 1b were tested, but for all other groups only one group of each rearing regime was used. Each individual was tested only once. Hatched bar or hatched part of a bar indicates fish kept together with siblings; open bar or open part of a bar indicates isolated fish without any contact with sibling odor; circles in an open bar indicate sibling-scented water was supplied to isolated fish. "Free swim." indicates time when fish had filled their swim bladder and were free swimming.

with separate compartments. These compartments were supplied with 20 ml/min of aerated tap water and 20 ml/min of sibling-scented water taken from the trough containing the 300 family A eggs and later fry. These eggs gave test groups 3a and 3b (rearing regimes 3a and 3b in Figure 1). Fish supported to rearing regime 3a were exposed to sibling odors up to the day of testing. At 15–16 months after fertilization, the supply of sibling odors was stopped to seven fish, giving rearing regime 3b and, the group was tested two months later as group 3b (median 56 days; range 52–60).

At the same time that the supply of sibling odor was terminated to the fish of groups 3b and 4b, 14 fish from the communal holding tank were individually isolated for about two months (median 56 days; range 50–60) in compartments equivalent to the others but only supplied with tap water (40 ml/min). This rearing regime is called 1-isol (Figure 1) and the group tested is referred to as group 1-isol.

The fish hatched in early January (2.5 months after fertilization), and in March the fish isolated since fertilization were transferred to equivalent troughs with larger compartments (polyethylene beakers), but still supplied with the same water qualities at the same flow rate.

The fish were fed 1–2% of their body fresh weight per day (Astra-EWOS F139H). The automatic light/dark regimen reflected conditions at latitude 51°N, with the length of the light/dark periods being automatically adjusted to take seasonal variations into account.

Testing Apparatus. One individual at the time was tested in the standard fluviarium (Höglund, 1961) according to the procedure described by Olsén and Höglund (1985) and Olsén (1986, 1989). Each fish was tested only once. The fish was placed in the test area at least 30 min before the initial test period. Equal amounts of water (340–360 ml/min) from each aquarium (Aq 1 and Aq 2, Figure 2 in Olsén, 1989) were pumped through silicone tubes to opposite lateral halves of the fluviarium. Every 90 min for 720 min the supply was switched automatically from one side to the other. Between tests, both aquaria and the test area in the fluviarium were brushed with 95% ethanol and water. Ethanol and water were also pumped through the tubes and electromagnetic valves.

Two 20-W halogen lamps equipped with a red glass filter (RG 780, Melles Grio), with no transmission of wavelengths shorter than 750 nm (and out of range of the visual system of salmonid fish; Bone et al., 1995), were used as the light source for the video camera. This infrared light was reflected by a glass mirror and a sheet of aluminum foil through a plate of opalescent white glass situated immediately below the bottom of the test area. On the video monitor this arrangement gave a light background, without reflections, against which the fish was readily detected.

The video camera (4.8 mm lens) was placed in the plywood hood, 33 cm above the test area. The camera was connected to a monitor and a computer in an adjacent room.

The position of the fish was recorded once every 2 sec by means of a video-computer-based image analysis system. This system has previously been described for studies of fish behavior, locomotor activity measurements (Winberg et al., 1993), and preference/avoidance responses to chemical substances (Bjerselius et al., 1995). These authors have described the hardware used in the present study. The software used in this image analysis system consisted of the program EthoVision (Noldus Information Technology bv, Wageningen, The Netherlands).

In this system, the data acquisition procedure was based on the following algorithm. A picture of the empty test area was digitized. Thereafter, a picture of the test area with the fish was made. These two pictures were subtracted from each other, leaving the image of the fish and the noise. The noise was filtered

by a threshold filter, followed by transformation to a binary image. Irrelevant objects (e.g., feces) were deleted by testing objects against a criterium of size. In the present study the computer calculated the number of observations in each lateral half of the test area for each 30 min period of the trial.

Procedure. Each fish was only tested once in the fluviarium, but odor donors may have been used more than once in different situations. Each test started at 18:00 hr and ended at 06:00 hr the next morning in order to avoid ordinary working hours in the lab. All tests were run in darkness. Fish from the different rearing conditions were tested for their preference when given a simultaneous choice between water scented by siblings and nonsiblings, respectively. In a control test, unscented water from the two donor aquaria was added to opposite lateral halves of the fluviarium.

Experiment 1. All tests were performed between February 15 and March 31. Fish reared in isolation with a supply of only water (group 2 from rearing regime 2) or a supply of sibling-scented water (groups 3a and 4a), and fish reared together with siblings (group 1a) were tested. Fish of the different groups weighed the same (mean $\pm$ SD: 6.6 $\pm$ 2.4 g; $N = 33$). Twenty char were placed as odor donors in each aquaria. The weights of the odor donors were matched just before they were placed in the donor aquaria so that the difference in total weight between the donor groups was always less than 10%. The water temperature increased during the testing period from 8.3 to 12.1°C.

Experiment 2. All tests were performed between May 1 and July 1. The supply of sibling-scented water to isolated fish was removed (rearing regimes 3b and 4b). Moreover, the effect of isolation later in life was tested by transferring individual fish from communal aquaria to separate compartments, giving rearing regime 1-isol. The fish were reared under these conditions for ca. 2 months (median 56 days, range 52–60) after which they were tested as group 1-isol for their preference when given a simultaneous choice between water scented by siblings and nonsiblings, as described above. Two groups of fish reared with siblings (group 1b) were also tested. The mean weight of all test fish used was 10.7 $\pm$ 3.9 g ($N = 32$). There were 15 odor donors in each donor aquarium. An increase in size of the fish justified the use of fewer odor donors than in experiment 1. The water temperature range was 12.0–14.0°C.

Data Analysis. A fish's preference for either water quality was determined on the basis of the number of observations in each. A reaction value (Rv) for a test was calculated as stated in Olsén (1986) from the following equation:

$$Rv = [(N_1 - N_2)/(N_1 + N_2)] \times 100$$

where N_1 and N_2 represent the number of observations in each half of the test area supplied with water from donor aquaria 1 and 2, respectively. Siblings and nonsiblings were always placed in donor aquaria 1 and 2, respectively. Thus, an attraction towards sibling-scented water is indicated by a positive Rv. The

number of observations ($N_1 + N_2$) in each test was 14,400 (8 × 1800). (For further details see Olsén and Höglund, 1985; Olsén, 1986.)

The arithmetic means of reaction values from identical tests are designed *MRv*. Confidence limits (CL) of *MRv*s were calculated using *t* statistics. The reaction was said to deviate from an indifferent reaction, i.e., from *MRv* = 0, if *MRv* ± 95% CL did not include *MRv* = 0. Kruskal-Wallis analysis was used as an initial test to reveal differences between groups within an experiment. When the Kruskal-Wallis analysis indicated differences between groups, the Mann-Whitney U test (two-tailed) was used to compare the reaction of two groups (Siegel, 1956).

RESULTS

Control Tests. The test fishes did not prefer either water current of unscented water from the two donor aquaria being added to opposite lateral halves of the fluviarium, i.e., the total reaction did not deviate from *MRv* = 0 (*MRv* ± 95% CL: −2.9 ± 5.0) (Figure 2).

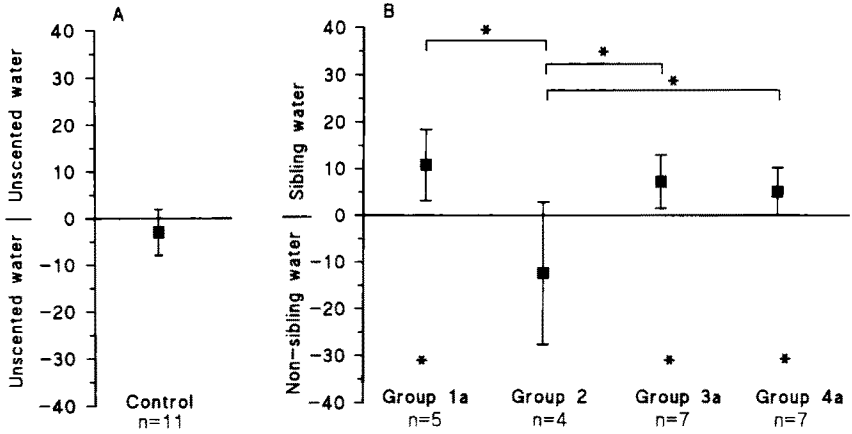

FIG. 2. Reaction of one char at a time during simultaneous choice between two types of water in the fluviarium. (A) Unscented water supplied to both sides, (B) water scented by siblings to the test fish and nonsiblings supplied to opposite sides. Reactions are given as mean reaction values, *MRv* ± 95% CL, for each group (for description of groups see Figure 1 and the text). The reaction shown by a group was said to deviate from an indifferent reaction, i.e., from *MRv* = 0, if *MRv* ± 95% CL did not include *MRv* = 0. Number of fish tested is indicated below each group. A significant difference between groups is denoted by asterisks and a significant difference from the control by asterisks in connection to group affiliation (Mann-Whitney U test). $*P < 0.05$.

Experiment 1. The reactions of char from groups 1a, 3a, and 4a were significantly different from the results of the control test (Figure 2). Kruskal-Wallis analysis revealed that there were differences in reactions between groups 1a, 2, 3a, and 4a ($H = 8.62$, $df = 3$, $0.02 < P < 0.05$).

Char raised communally with siblings (group 1a) preferred water scented by siblings to water scented by nonsiblings (*MRv* ± 95% CL: +10.8 ± 7.6) (Figure 2). Individuals reared in isolation without any supply of sibling-scented water (group 2) showed no preference for sibling- or nonsibling-scented water (*MRv* ± 95% CL: −12.4 ± 15.2) (Figure 2). However, fish that were raised in isolation in compartments provided with sibling-scented water (group 3a) preferred water scented by siblings to water scented by nonsiblings (*MRv* ± 95% CL: +7.2 ± 5.7) (Figure 2). Char that were reared in such compartments but with a supply of siblings odor from free swimming fish (group 4a) exhibited an attraction that approached significance (*MRv* ± 95% CL: +5.1 ± 5.1) (Figure 2).

Individuals raised in isolation in compartments supplied with sibling-scented water since fertilization (group 3a) did not differ from fish that were exposed to sibling odors since they were free swimming (group 4a, Figure 2). However, fish raised with siblings (group 1a) as well as fish raised in separate compartments supplied with sibling odor, either during the whole rearing period (group 3a) or since start of free swimming (group 4a), differed significantly ($P = 0.016$, $P = 0.042$ and $P = 0.024$, respectively) from the char that had been raised in isolation without any supply of sibling-scented water (group 2).

Experiment 2. The reaction of group 1 char (two different groups of 1b in Figures 3 and 4) and group 1-isol (Figure 4) were significantly different from the controls. Kruskal-Wallis analysis revealed differences in reaction between groups 1b, 3b, and 4b ($H = 8.51$, $df = 2$, $0.01 < P < 0.02$) (Figure 3). There was a significant difference in reaction between groups 1b and 4b (Figure 3).

Fish that had been reared in separate compartments supplied with sibling-scented water during the entire rearing period and those that had been reared in such compartments with a supply of sibling odors since they were free swimming showed no significant preference for water scented by siblings after being deprived of the experience of sibling-scented water for two months (*MRv* ± 95% CL for group 3b: +2.2 ± 7.9; and for group 4b: −2.0 ± 4.8) (Figure 3).

No difference in reaction was observed between the fish that remained in the trough with siblings (group 1b) and the fish that previously had been reared in this group but were removed to separate compartments two months prior to testing (group 1-isol) (Figure 4). Both groups showed attraction to sibling-scented water (*MRv* ± 95% CL for group 1b: +10.0 ± 7.6; and for group 1-isol: +12.0 ± 6.7).

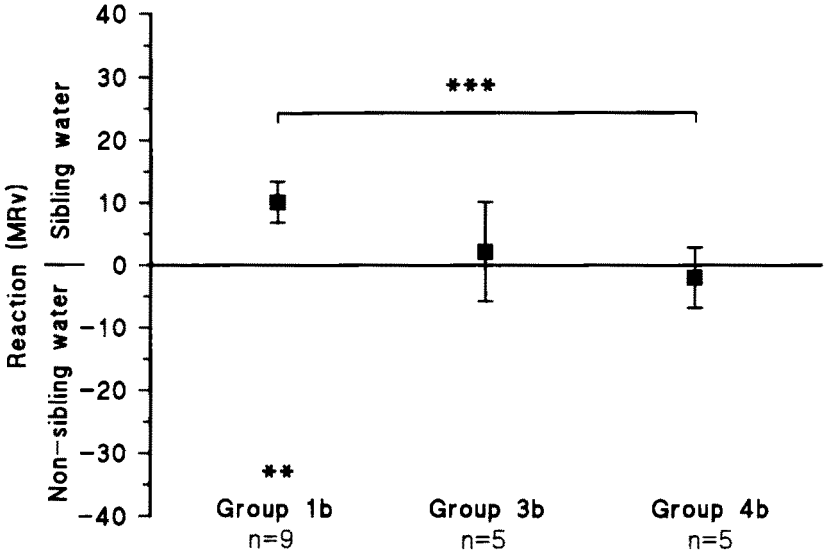

FIG. 3. Reaction of one char at a time during simultaneous choice between water scented by siblings to the test fish and nonsiblings in the fluviarium. Isolated fish preexposed to sibling-scented water were reared for two months without a supply of odors (groups 3b and 4b). For a description of groups see Figure 1 and the text. Reactions are given as described in Figure 2. The number of fish tested is indicated below each group. A significant difference between groups is denoted by asterisks and a significant difference from the control by asterisks in connection to group affiliation (Mann-Whitney U test). $^{**}P < 0.01$; $^{***}P < 0.001$.

DISCUSSION

The present study suggests that communal rearing and social interactions were important for young Arctic char to remember sibling specific odors, at least in the sibling group studied. Individuals only preexposed to water scented by siblings since fertilization or since they were free swimming, but without social contact, lost their ability or motivation to discriminate between sibling and nonsibling odors after termination of the exposure. The results support our previous study in which we suggested that social interactions during the first period after hatching are important for development of sibling recognition in Arctic char (Winberg and Olsén, 1992). We do not know how long the char retain their memory of the odors of their siblings, but salmonids do have the capacity of long-term olfactory memory as, for example, the spawning migration to the home river after one to three years in the sea (Brannon, 1972; Hasler and

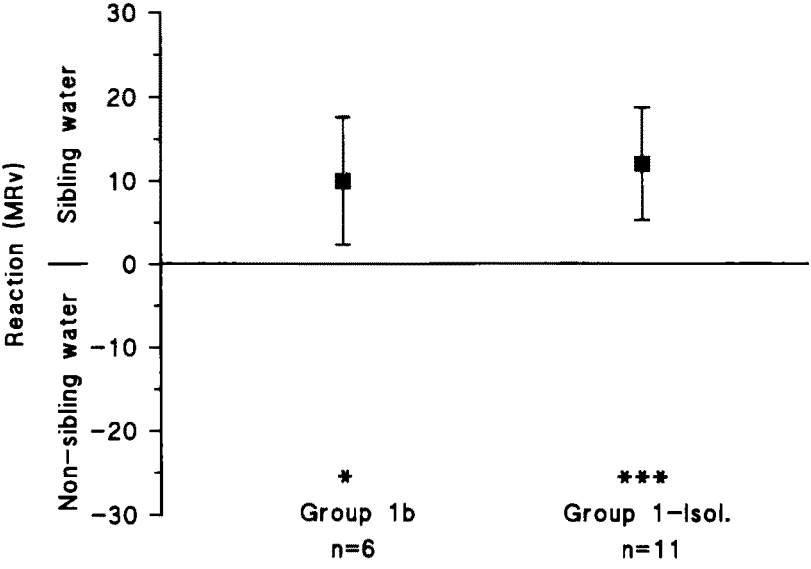

FIG. 4. Reaction of one char at a time during simultaneous choice between water scented by siblings to the test fish and nonsiblings in the fluviarium. Individuals were taken from sibling group A and reared in isolation for two months without a supply of sibling odors (group 1-isol). Test group 1b was not composed of the same fish as in group 1b of Figure 3. For description of groups see Figure 1 and the text. Reactions are given as described in Figure 2. The number of fish tested is indicated below each group. A significant difference between groups is denoted by asterisks and a significant difference from the control by asterisks in connection to group affiliation (Mann-Whitney U test). $*P < 0.05$; $***P < 0.001$.

Scholz 1983; Dodson, 1988). Odors from kin may be an important part of the home river bouquet (Nordeng, 1971; Stabell, 1984; Olsén, 1986).

In tadpoles of some species, learning of sibling odors may take place before hatching (Waldman, 1981; Blaustein and Waldman, 1992; Hepper and Waldman, 1992). In these species individuals isolated as eggs were also able to discriminate between odors of siblings and nonsiblings, an ability that seams to have an important maternal component (Blaustein and O'Hara, 1982). In contrast to our observations with juvenile Arctic char, Van Havre and Fitzgerald (1988) observed that juvenile sticklebacks could discriminate between sibling- and nonsibling-scented water even though the test fish had been isolated since the egg stage. Differences in the mechanisms behind sibling discrimination may reflect differences in natural history traits (O'Hara and Blaustein, 1988).

Grafen (1990) and Barnard (1990) proposed that most studies indicating the existence of kin discrimination fail to demonstrate true kin discrimination, but possibly only species recognition. Previous experiments have demonstrated that juvenile Arctic char are attracted to odors from conspecifics, but not to three other salmonid species (Höglund and Åstrand, 1973; Höglund et al., 1975). They are, in addition, able to discriminate between their own population and another sympatric population (Olsén, 1986) and also between odors from their own unfamiliar siblings and another sibling group from the same population (Olsén, 1989; Winberg and Olsén, 1992). These results give support to the view that juvenile Arctic char are not only able to recognize their own species odor ("pheromone," Karlsson and Luscher, 1959), but also intraspecific variation in odors, which may be a basis for kin recognition.

There are some experiments suggesting that intraspecific odors of salmonids are involved in nepotism (Brown and Brown, 1993a,b). Whether this is due to "true kin recognition" (Grafen, 1990), familiarity, or imprinting to genetically determined intraspecific odors (Winberg and Olsén, 1992), the result may be the same. Recent results with Atlantic salmon, rainbow trout (Brown and Brown, 1993a,b), and brown trout (Olsén, Järvi and Löv, accepted manuscript) have revealed that the aggressiveness in groups of siblings is significantly less than in mixed groups with both siblings and nonsiblings. The authors suggested that kin-biased behavior increased an individual's inclusive fitness, which, according to Hamilton (1964), includes the survival and reproductive success of an individual and its close relatives. Results from Brown and Brown (1993a,b) supported this hypothesis. The authors also observed that the territories were smaller and the growth rate better in pure sibling groups. Brown and Brown (1993a,b) suggested that these benefits of kin-biased behavior may increase the probability of survival.

The sibling group of Arctic char followed in the present study belongs to a lacustrine population with the entire life cycle in Lake Hornavan, Swedish Lapland. Individuals of Arctic char are, at least in lakes, thought to remain together in schools (Aass, 1970; Klemetsen and Grotnes, 1980). Thus kin-biased behavior should not be connected to territorial behavior, as suggested in young specimens of the above-mentioned three salmonid species. If, however, schools of Arctic char are composed of siblings, altruistic behavior towards kin and cooperation within the group may increase an individual's inclusive fitness (cf., Hamilton, 1964). Young individuals of lacustrine Arctic char, including the present population studied, may, however, show high degrees of aggressiveness in small experimental groups (Winberg et al., 1991, 1992), indicating that there is a high degree of plasticity in their occurrence and behavior. Changes in behavior within a group from schooling to agonistic or the reverse may reflect the local distribution of food and predator risk, as has been suggested for juvenile

chum salmon (*Oncorhynchus keta*) (Ryer and Olla, 1991). Fish presented with a defensible source of food abandon schooling and instead fight for access to the source. Kin-biased behavior with positive effects on the individual's inclusive fitness may still work, although schooling is abandoned in favor to territorial defense. The aggressiveness in pure sibling groups of juvenile Lake Hornavan char was significantly lower than in groups of mixed relationship (Olsén and Järvi, unpublished data).

The selfish herd theory proposed by Hamilton (1971) suggests that individuals in a school act in a selfish way to decrease the risk of predation. It may, however, be possible that individuals in schools of kin act in a cooperative way, not observed in groups of unrelated individuals, that increases the members' chances to avoid being eaten by predators (Waldman, 1991). We still do not know, however, if char and other fish species prefer to school with related individuals, as has been demonstrated for tadpoles of some species (Waldman, 1991). The possible preference of juvenile Arctic char to school with kin has to be examined before an analysis of the significance of kinship among school members in relation to predation and survival in general can be tested.

Present knowledge of the behavioral ecology of different local populations or stocks of Arctic char, especially during the first year of life, is scarce and further investigation is needed (Johnson, 1980; Noakes, 1980). We do not know if the present results with the sibling group tested are representative of the population. The high plasticity in behavior within the species is further stressed by the observation that significant population differences in aggressiveness (Olsén and Karlsson, 1990) and foraging behavior (Skúlason et al., 1993) are present during the same experimental conditions.

In summary, communal rearing, which included social interactions, was probably obligatory for long-term memory of sibling-specific odors in the group used in the present study. Social interactions may be obligatory for sibling discrimination in juvenile Arctic char in general. The functions of and mechanisms behind sibling recognition ability and behavioral discrimination in Arctic char have to be investigated further in the context of a plastic species with diverse behavior at individual and local population levels.

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SEX PHEROMONE OF TOMATO PEST *Scrobipalpuloides absoluta* (LEPIDOPTERA: GELECHIIDAE)

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Abstract—The sex attractant of *Scrobipalpuloides absoluta* females is a 90:10 mixture of (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate and (3*E*,8*Z*)-3,8-tetradecadien-1-yl acetate. Tetradecadienyl acetates bearing 8*Z*,11*Z*; 3*E*,8*Z*; and 3*E*,11*Z* double bonds were synthesized by stereospecific procedures; the mass spectral and gas chromatographic properties of the 3*E*,8*Z* isomer were found to be congruent with those of the tetradecadienyl acetate from *S. absoluta*. In wind tunnel bioassays, a 10:1 mixture of synthetic (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate and (3*E*,8*Z*)-3,8-tetradecadien-1-yl acetate was highly attractive to *S. absoluta* males. Interestingly, the presence of (8*Z*,11*Z*)-8,11-tetradecadien-1-yl acetate (10%) inhibited the response to (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate completely.

Key Words—Lepidoptera, Gelechiidae, *Scrobipalpuloides absoluta*, tomato, pest, pheromone.

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INTRODUCTION

We found recently that the female sex pheromone of *Scrobipalpuloides absoluta* (Lepidoptera: Gelechiidae), a devastating tomato pest in South America, consists of two components. The major component, which represents about 90% of the volatile material found in the sex gland of calling females, was identified as (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate (**1**, Figure 1) (Attygalle et al., 1995; 1996). The minor constituent (10%), was characterized partially by GC-MS as a tetradecadienyl acetate. However, the minute amount of natural material available precluded a full structural assignment by conventional methods.

In order to identify the diene acetate, we decided to synthesize the three possible dienes (**2–4**, Figure 1) with double-bond positions and configurations corresponding to those of the major pheromone component, (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate, and to compare the properties of the synthetic samples with those of the natural material. Results of these experiments, and subsequent bioassays, established the pheromone as a mixture of (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate (**1**) and (3*E*,8*Z*)-3,8-tetradecadien-1-yl acetate (**3**).

METHODS AND MATERIALS

NMR spectra were recorded on a Unity-200 (^{1}H, 200 MHz, Varian), a Unity-400 (^{1}H NMR, 400 MHz; ^{13}C NMR, 100.6 MHz, Varian), and a Unity-

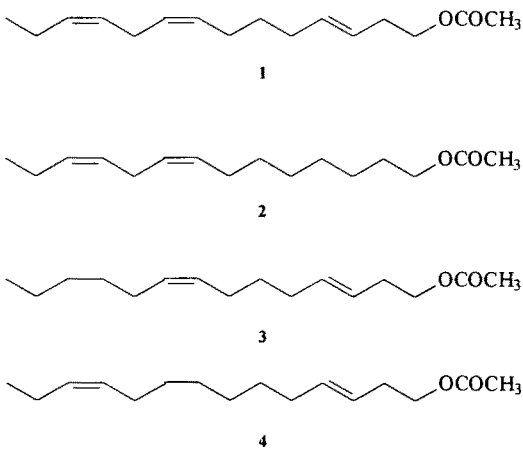


FIG. 1. Structures of compounds **1–4.**

500 instrument (^{1}H NMR, 500 MHz; ^{13}C NMR, 125.7 MHz, Varian) as $CDCl_3$ solutions at room temperature. Chemical shifts, given in parts per million, are expressed as δ values measured from the residual $CHCl_3$ signal (7.26 ppm). Vapor-phase infrared spectra were recorded using a Hewlett-Packard (HP) 5965A IRD coupled to a HP 5890 GC. Electron impact (70 eV) spectra were obtained using a HP 5890 GC coupled to an ion trap detector (ITD 800, Finnigan) or a mass selective detector (HP 5970 MSD). Fast atom bombardment (FAB, in a glycerin matrix) mass spectra were obtained using a ZAB-Q (VG) instrument. For GC analysis a Supelco fused-silica capillary column (30 m × 0.25 mm) coated with SE-54 (0.25 μm) fitted in a HP 5890 GC equipped with a flame-ionization (FID) detector was used. Synthetic samples (hexane, ca. 1 mg/ml) were injected in split mode using a temperature program of 60°C for 4 min, 10°C/min to 270°C, and held for 20 min. Column chromatography was run on silica gel (Merck, H 60) and reactions were monitored by TLC on Baker-flex Silica gel IB2-F plates (J. T. Baker) and on Silufol TLC plates (Kavalier, Sázava, Czech Republic). Silver-nitrate column chromatography was done on silica gel impregnated with $AgNO_3$ (20% of $AgNO_3$ on silica gel Merck H 60).

Biological Material

Pheromone extracts were prepared as described previously (Attygalle et al., 1996).

Chemicals

Butyllithium (1.6 M and 2.5 M solutions in hexane), 1-(tetrahydropyran-2-yloxy)-3-butyne [2-(3-butynyloxy)tetrahydro-2*H*-pyran], $LiAlH_4$, $NaBH_4$, borane–methylsulfide complex, $Cu(I)Br \cdot Me_2S$ complex, and *p*-toluenesulfonyl chloride were from Aldrich Chemical Co. (Milwaukee, Wisconsin), and bromine, ethylenediamine, nickel(II) acetate, and triphenylphosphine from Fluka Chemical Co. (Ronkonkoma, New York); they were used as purchased. 1-(Tetrahydropyran-2-yloxy)-4-pentyne (**5**) (Robertson, 1960) and 1-bromo-6-(tetrahydropyran-2-yloxy)-hexane (**20**) (Kang et al., 1985; Miyashita et al., 1977) were prepared according to published procedures. 1,3-Dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU, Aldrich Chemicals) was dried over molecular sieves.

(8Z,11Z)-8,11-Tetradecadienyl Acetate (2)

This compound was synthesized according to Scheme 1. Compounds **5–9** were synthesized according to procedures described previously for the synthesis of (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate (**1**) (Attygalle et al., 1995; 1996).

Magnesium turnings (30 mg, 1.28 mmol) were warmed, stirred for 1 hr under dry argon, and covered with dry THF (100 μl). Ten drops of a solution of (4Z,7Z)-1-bromo-4,7-decadiene (**9**) (200 mg, 0.925 mmol) in dry THF (3 ml) were added, and the mixture was brought to reflux. After the reaction started, the remainder of the bromide solution was added dropwise at 0°C over a period of 1.5 hr, and the mixture was stirred for 2 hr at room temperature.

Dry THF (3 ml) was treated with trimethylsilyl iodide (260 mg, 185 μl, 1.30 mmol) at room temperature and stirred for 15 min to form 4-trimethylsilyloxy iodide (**11**) (Jung and Lyster, 1977). The reaction mixture was treated with a solution of $CuCN \cdot (LiCl)_2$ in THF (1 M, 100 μl), cooled to −40°C, and the solution of Grignard reagent **10** described in the preceding paragraph was then added over a period of 30 min (Tamura and Kochi, 1971; Lipshutz et al., 1984). The mixture was stirred at −40 to −20°C for 1 hr, followed by an additional 1 hr at room temperature. The reaction was quenched with NH_4Cl solution and ammonia mixture (2 : 1 v/v), and the product was extracted with hexane–ether (1 : 1) mixture (R_f = 0.60, 19 : 1 hexane–ethyl acetate, v/v). The crude product was dissolved in THF (6 ml), and deprotected with tetrabutylammonium fluoride solution in THF (1 M, 2 ml). The alcohol formed was acetylated with Ac_2O–pyridine mixture (1 : 10 v/v). Flash chromatographic purification afforded (8Z,11Z)-8,11-tetradecadien-1-yl acetate **2** (80 mg) in 34% yield based on the starting bromide **9.**

^{1}H NMR (400 MHz) δ: 5.36 (m, 4H, CH=CH-8, 9, 11, 12), 4.05 (t, J = 6.7, 7.0 Hz, 2H, CH_2-1), 2.77 (dd, J = 6.4, 6.4 Hz, 2H, =CH-CH_2-CH=), 2.06 (m, 4H, CH_2-7, 13), 2.04 (s, 3H, -$COCH_3$), 1.60 (tt, J = 7.3, 7.3, 7.6, 7.6 Hz, 2H, CH_2-6), 1.4-1.2 (m, 8H, CH_2-2, -3, -4, -5) 0.97 (t, J = 7.3, 7.6 Hz, 3H, CH_3-14). ^{13}C NMR (100.6 MHz) δ: 171.2 (C=O), 131.7 (=CH-12), 130.0 (=CH-11), 128.0 (=CH-9), 127.3 (=CH-8), 64.6 (CH_2-1), 29.5 (CH_2-2), 29.1 (CH_2-3, 4), 28.6 (CH_2-5), 27.2 (CH_2-6), 25.9 (CH_2-7), 25.5 (CH_2-10), 21.0 (CH_3C=O), 20.5 (CH_2-13), 14.3 (CH_3). MS [EI MSD, m/z (%)] 252 (M^+, 2), 209 (M^+ − 43, 1), 192 (M^+ − 60, 23), 163 (4), 149 (8), 135 (14), 121 (38), 110 (19), 107 (19), 95 (39), 93 (40), 82 (26), 81 (57), 79 (66), 67 (100), 55 (38), 43 (93), 41 (50). IR (gas phase, cm^{-1}) 3017 (cis=C−H str), 2936 (CH_2), 1761 (C=O), 1233, 1040 (−O−).

(3E,8Z)-3,8-Tetradecadienyl Acetate (3)

1-(Tetrahydropyran-2-yloxy)-4-decyne (13). A solution of 1-(tetrahydropyran-2-yloxy)-4-pentyne (**5**) (0.66 g, 3.90 mmol) in THF (3 ml) was metallated with n-butyllithium solution (2.5 M) in hexane (1.87 ml, 1.2 eq.), and a solution of n-pentyl bromide (0.725 ml, 1.5 eq.) in DMPU (2 ml) was added. This procedure gave 0.54 g (58% yield) of compound **13.**

^{1}H NMR (200 MHz) δ: 4.60 (m, 1H, CH-2′), 3.65 (m, 4H, CH_2-1, 6′),

2.26 (tt, J = 7.0, 7.0, 2.0, 2.0 Hz, 2H, CH_2-3), 2.13 (tt, J = 7.0, 7.0, 2.5, 2.5 Hz, 2H, CH_2-6), 1.77 (tt, J = 6.8, 6.8, 7.0, 7.0 Hz, 2H, CH_2-2), 1.33 (m, 6H, CH_2-7, 8, 9), 1.52 (m, 6H, CH_2-3′, 4′, 5′), 0.89 (t, J = 7.0 Hz, 3H, CH_3). MS [FAB, m/z(%)] 237 ($M^+ - 1$), 85 (100). IR (CCl_4, cm^{-1}) 2229, 1322, 1331, 1201, 1138, 1121, 1034.

*(4Z)-1-(Tetrahydropyran-2-yloxy)-4-decene (**14**).* 1-(Tetrahydropyran-2-yloxy)-4-decyne (**13**) (0.5 g, 2.1 mmol) was hydrogenated in ethanol (20 ml) over *P*2-Ni, formed from nickel acetate (75 mg) deactivated with ethylenediamine (0.2 ml) for 4 hr (Brown and Ahuja, 1973). After chromatography on silica gel, the product (**14**) (0.43 g) was obtained in 85% yield. GC (R_t): starting material **13,** 22.18 min, 0.4%; desired product **14,** 21.59 min, 97.9%; the 4*E* isomer of **14,** 21.75 min, 1.7%.

^{1}H NMR (200 MHz) δ: 5.37 (m, 2H, CH=CH-4, 5), 4.58 (m, 1H, CH-2′), 3.60 (m, 4H, CH_2-1, 6′); 2.12 (m, 2H, CH_2-6); 2.04 (m, 2H, CH_2-3); 1.16 (tt, 2H, J = 6.8, 6.8, 7.0, 7.0 Hz, CH_2-2); 1.4–1.9 (m, 6H, CH_2-3′, 4′, 5′); 1.29 (m, 6H, CH_2-7, 8, 9); 0.88 (t, 3H, J = 7.0 Hz, CH_3). IR (CCl_4, cm^{-1}) 3007, 1653, 1201, 1138, 1120, 1034. MS [FAB, m/z (%)] 239 ($M^+ - 1$), 85 (100).

*(4Z)-1-Bromodec-4-ene (**15**).* To an ice-cool solution of triphenylphosphine (0.72 g, 2.74 mmol) and bromine (0.41 g, 2.6 mmol) in CH_2Cl_2, a solution of (4*Z*)-1-(tetrahydropyran-2-yloxy)-4-decene (**14**) in CH_2Cl_2 (0.41 g, 1.71 mmol) was added dropwise (Bestmann and Gunawardena, 1992). The usual work-up (Attygalle et al., 1996) gave the product **15** in 90% yield (0.33 g).

^{1}H NMR (200 MHz) δ: 5.42 (dtt, J = 11.0, 7.0, 7.0, 1.0, 1.0 Hz, 2H, CH=CH-4, 5), 3.41 (t, J = 6.6, 6.6 Hz, 2H, CH_2-1), 2.20 (dt, J = 7.0, 7.0, 7.0 Hz, 2H, CH_2-3), 2.04 (dt, J = 6.5, 6.5, 6.5 Hz, 2H, CH_2-6), 1.91 (tt, J = 6.5, 6.5, 6.5 Hz, 2H, CH_2-2), 1.30 (m, 6H, CH_2-7, 8, 9), 0.89 (t, J = 7.0 Hz, 3H, CH_3). MS [EI MSD, m/z (%)] 220 (M^+, 8), 218 (M^+, 8), 164 (12), 162 (12), 150 (23), 148 (23), 135 (8), 109 (12), 97 (44), 95 (14), 83 (55), 82 (14), 81 (37), 79 (15), 70 (21), 69 (100), 67 (39), 56 (29), 55 (93), 54 (20), 43 (21), 42 (20), 41 (92), 39 (30). IR (CCl_4, cm^{-1}) 3008 (*cis* CH=CH), 1246 (RCH_2CH_2Br), 649, 566 (C-Br).

*(8Z)-1-(Tetrahydropyran-2-yloxy)-8-tetradecen-3-yne (**16**).* 1-(Tetrahydropyran-2-yloxy)-3-butyne (0.30 g, 1.94 mmol) was metallated at 0°C with *n*-butyllithium (2.0 M) hexane solution (1.0 ml, 1.0 eq.). The lithium acetylide that formed was alkylated with the bromide **15** (0.20 g, 0.92 mmol), using 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidine (DMPU) as the base. This reaction afforded 214 mg of a mixture containing the desired product **16,** 1-(tetrahydropyran-2-yloxy)-3-butyne (one of the starting materials; 29%), and a nonpolar impurity identified as (8*Z*)-1,8-tetradecadien-4-yne (**19**) (28%). The impurities were removed from compound **16** (52% yield based on **15**) by repeated column chromatography.

^{1}H NMR (200 MHz) (**16**) δ: 5.35 (dtt, J = 11.0, 7.0, 7.0, 1.0, 1.0 Hz, 2H, CH=CH-8, 9), 4.64 (m, 1H, CH-2′), 3.58 (m, 4H, CH_2-1, 6′), 2.46 (tt, J = 7.5, 7.5, 2.5, 2.5 Hz, 2H, CH_2-2), 2.14 (tt, J = 7.0, 7.0, 2.5, 2.5 Hz, 2H, CH_2-5), 2.10 (m, 2H, CH_2-7), 2.04 (dt, J = 6.5, 6.5, 7.0 Hz, CH_2-10), 1.29 (m, 6H, CH_2-11, 12, 13), 1.9–1.4 (m, 8H, CH_2-6, 3′, 4′, 5′), 0.89 (t, J = 7.0 Hz, 3H, CH_3). MS [EI MSD, m/z (%)] (**16**) 133 (12), 107 (10), 85 (100), 67 (15), 55 (8). MS [FAB, m/z] 293.2 (M^+). IR (CCl_4, cm^{-1}) (**16**) 3007 (*cis*-CH=CH), 2232 (acetylenic), 1403, 1201, 1184, 1137, 1122, 1034 (CH_n—O—).

^{1}H NMR (200 MHz) [(8*Z*)-1,8-tetradecadien-3-yne(**19**)] δ: 5.78 (ddt, J = 22.5, 10.5, 2.2, 2.2 Hz, 1H, =CH-2), 5.54 (dd, J = 22.5, 2.7 Hz, 1H, $=CH_2$-1a), 5.36 (dd, J = 10.5, 2.2 MHz, 1H, CH_2-1b), 5.36 (dtt, J = 11.0, 7.0, 7.0, 1.0, 1.0 Hz, 2H, CH=CH-8, 9), 2.31 (dt, J = 2.1, 7.3, 7.3 Hz, 2H, CH_2-5), 2.15 (dt, J = 7.0, 7.3, 7.3 Hz, 2H, CH_2-10), 2.03 (dt, J = 6.4, 6.4, 6.7 Hz, 2H, CH_2-7), 1.59 (tt, J = 7.0, 7.0, 8.0, 8.0 Hz, 2H, CH_2-6), 1.30 (m, 6H, CH_2-11, 12, 13), 0.89 (t, J = 7.0 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$) (**19**) δ: 131.1 (=CH-9), 126.4 (=CH-8), 125.5 ($=CH_2$-1), 117.6 (=CH-2), 90.9 (≡C-4), 79.5 (≡C-3), 31.5 (CH_2-12), 29.4 (CH_2-10), 28.7 (CH_2-7), 27.2 (CH_2-11), 26.3 (CH_2-6), 22.6 (CH_2-5), 18.8 (CH_2-13), 14.1 (CH_3-14). MS [EI MSD, m/z (%)] (**19**) 161 (M^+ −29, 5), 133 (35), 119 (25), 105 (50), 91 (100), 79 (35), 67 (30), 41 (50). IR (CCl_4, cm^{-1}) (**19**) 3101 ($=CH_2$), 3009 (*cis*-CH=CH and $=CH_2$), 2227, 2206 (acetylenic), 1833, 972, 913 (vinyl).

(Z)-8-Tetradecen-4-ynol (17). Tetrahydropyranyl ether **16** was deprotected with Dowex 50WX8 (40 mg) to form the alcohol **17** (107 mg, 56% yield based on the starting bromide 15).

^{1}H NMR (200 MHz) δ: 5.35 (dtt, J = 11.0, 7.0, 7.0, 1.0, 1.0 Hz, 2H, CH=CH-8,9), 3.68 (dt, J = 6.5, 5.6, 5.6 Hz, 2H, CH_2-1), 2.44 (tt, J = 6.5, 6.5, 2.5, 2.5 Hz, 2H, CH_2-2), 2.17 (tt, J = 7.0, 7.0, 2.5, 2.5 Hz, 2H, CH_2-5), 2.12 (m, 2H, CH_2-7), 2.04 (m, 2H, CH_2-10), 1.76 (bt, J = 6.5, 6.5 Hz, 1H, OH), 1.55 (tt, J = 4 × 7.2 Hz, 2H, CH_2-6), 1.30 (m, 6H, CH_2-11, 12, 13), 0.89 (t, J = 7.0 Hz, 3H, CH_3). MS [EI MSD, m/z (%)] 179 (M^+-29, 8), 163 (21), 151 (15), 133 (53), 121 (38), 107 (87), 95 (66), 93 (66), 91 (80), 81 (66), 79 (88), 67 (87), 55 (100), 41 (100), 29 (29). IR (CCl_4, cm^{-1}) 3636, 3588 (OH), 3006 (*cis*-CH=CH), 2227 (acetylenic), 1654 (C—O).

(3E,8Z)-3,8-Tetradecadien-1-yl Acetate (3). (8*Z*)-Tetradecen-4-ynol (**17**) (21 mg, 0.1 mmol) was reduced with $LiAlH_4$ (20 mg, 0.5 mmol) in dry diglyme (0.5 ml) at 120°C for 3 hr. The alcohol (**18**) that formed was acetylated with Ac_2O (0.1 ml) and dry pyridine (0.5 ml) and the product was purified on $AgNO_3$ (20%) impregnated silica gel. This procedure afforded 18 mg (72% yield) of pure **3** (97% isomeric purity).

^{1}H NMR (500 MHz) δ: 5.51 (dtt, J = 15.3, 6.8, 6.8, 1.2, 1.2 Hz, 1H, =CH-3), 5.38 (dtt, J = 15.2, 6.6, 6.6, 1.2, 1.2 Hz, 1H, =CH-4), 5.35 (dtt

J = 11, 6.8, 6.8, 1.2, 1.2 Hz, 2H, CH=CH-8, 9), 4.07 (t, J = 6.9, 6.9 Hz, 2H, CH_2-1), 2.31 (dtdt, J = 3 × 6.8, 3 × 1.2 Hz, 2H, CH_2-2), 2.01 (tt, J = 4 × 7.0 Hz, 6H, CH_2-5, 7, 10), 2.04 (s, 3H, $COCH_3$), 1.60–1.20 (8H, m, CH_2-6, 11, 12, 13), 0.88 (t, J = 7.0, 7.0 Hz, 3H, CH_3). ^{13}C NMR (125.7 MHz) δ: 171.1 (C=O), 133.3 (=CH-3), 130.3 and 129.4 (=CH-8, 9), 125.3 (=CH-4), 64.1 (CH_2-1); 32.2 (CH_2-2), 32.0 (CH_2-6), 31.5 (CH_2-12), 29.4 (CH_2-7, 10), 27.2 (CH_2-11), 26.6 (CH_2-5), 22.5 (CH_2-13), 21.0 ($COCH_3$), 14.1, (CH_3-14). MS [EI, *m/z* (%)], 252 (M^+, 0.5), 192 (M^+ −60, 12), 163 (5), 138 (19), 124 (20), 121 (41), 107 (19), 95 (34), 93 (34), 82 (41), 80 (100), 79 (63), 67 (60), 55 (36), 43 (100), 41 (41). IR (CCl_4, cm^{-1}) 3006 (*cis*=C-H str), 1743 (C=O), 1653 (CH=CH), 1403, 1238, 1035 (C−O), 969 (*trans* CH=CH wag). IR (gas phase, cm^{-1}) 3011 (*cis*=C-H str), 2934 (CH_2), 1761 (C=O), 1231, 1037 (−O−), 967 (*trans* CH=CH wag).

(3E,11Z)-3,11-Tetradecadien-1-yl Acetate (4).

Starting from 1-butyne and 1-bromo-6-(tetrahydropyran-2-yloxy)-hexane (**20**), the (3*E*,11*Z*)-3,11-tetradecadien-1-yl acetate (**4**) was prepared by a synthetic route similar to that used for the preparation of (3*E*,8*Z*)-3,8-tetradecadien-1-yl acetate (**3**). The yield was 15% [17 mg; 96% isomeric purity (GC)] based on the starting material, 1-bromo-6-(tetrahydropyran-2-yloxy)-hexane (**20**).

^1H NMR (500 MHz) δ: 5.51 (dtt, J = 15.1, 6.8, 6.8, 1.2, 1.5 Hz, 1H, =CH-3), 5.35 (m, 3H, CH=CH-4, 11, 12), 4.06 (t, J = 6.8, 6.8 Hz, 2H, CH_2-1), 2.31 (dddt, J = 1.2, 1.2, 6.8, 6.8, 6.8 Hz, 2H, CH_2-2), 2.05 (s, 3H, $COCH_3$), 2.02 (m, 6H, CH_2-5, 10, 13), 1.36–1.24 (m, 6H, CH_2-6, 7, 8, 9), 0.95 (t, J = 7.3, 7.6 Hz, 3H, CH_3). ^{13}C NMR (100.6 MHz) δ: 171.1 (C=O), 133.6 (=CH-3), 131.6 (=CH-12), 129.3 (=CH-11), 125.0 (=CH-4), 64.1 (CH_2-1), 32.6 (CH_2-2), 31.9 (CH_2-6), 29.7 (CH_2-8), 29.3 (CH_2-7), 29.1 (CH_2-9), 29.0 (CH_2-10), 27.1 (CH_2-5), 21.0 ($COCH_3$), 20.5 (CH_2-13), 14.4 (CH_3-14). MS [EI MSD, *m/z* (%)] 192 (M^+-60, 6), 163 (6), 149 (9), 135 (12), 121 (16), 107 (16), 96 (28), 95 (37), 93 (25), 82 (50), 81 (60), 80 (35), 79 (36), 69 (33), 68 (64), 67 (97), 55 (45), 43 (100), 41 (67). IR (CCl_4, cm^{-1}) 3006 (=CH *cis* str), 1743 (C=O), 1238, 1036 (C−O), 969 (=CH *trans* wag). IR (gas phase, cm^{-1}) 3012 (*cis*=C-H str), 2934 (CH_2), 1761 (C=O), 1231, 1038 (−O−), 968 (=CH *trans* wag).

Wind-Tunnel Experiments

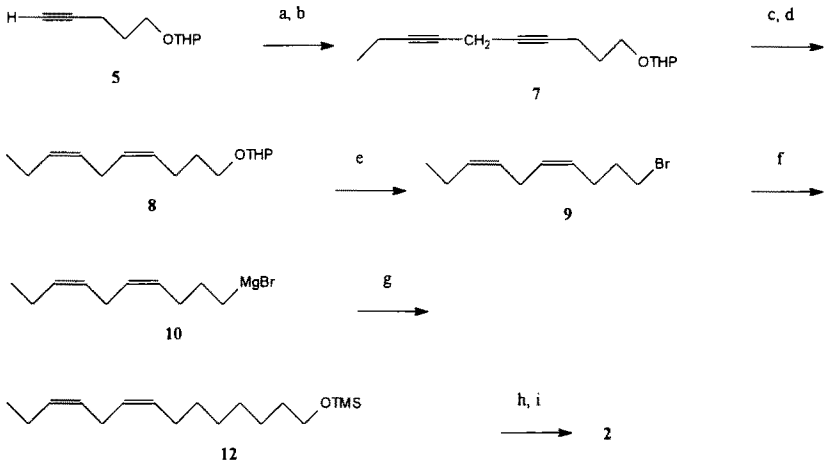
Synthetic samples of test compounds were applied as pentane solutions to rubber septa (cleaned by washing with CH_2Cl_2 for 20 hr in a Soxhlet apparatus), which were used as baits in the wind tunnel. The attractivity of baits loaded with either 100 ng of (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate (**1**), or each individual diene acetate (100 ng, **2–4**), or mixtures of 100 ng of **1** with 10 ng

of each diene acetate **2, 3,** and **4** were compared to that of three calling virgin females (1 to 4 days old) in a cage. The control baits were treated with 100 μl of hexane. The wind tunnel (3.8 × 0.50 m) was operated at a flux speed of 30 cm/sec. The landing platform was 1 m away from the "takeoff" platform. For each experiment, three males (1 to 3 days old) were placed on the takeoff platform and their behavioral responses were observed for 5 min. The test was repeated 10 times for each test sample.

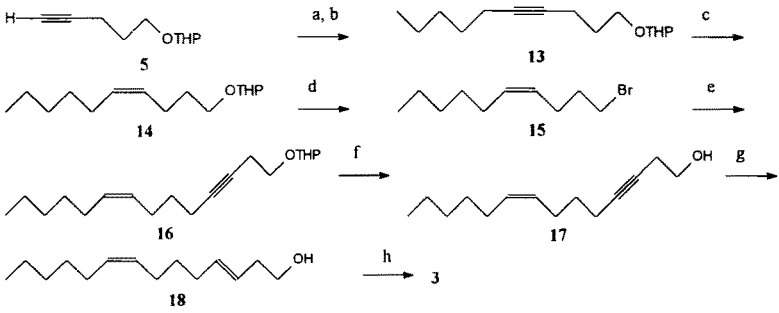
RESULTS AND DISCUSSION

In order to characterize the minor component of the *S. absoluta* pheromone, the dienes **2, 3,** and **4** were synthesized. (8*Z*,11*Z*)-8,11-Tetradecadienyl acetate (**2**) was synthesized according to the reaction shown in Scheme 1.

Intermediates **5–9** were synthesized according to procedures described previously for the synthesis of (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate (**1**) (Attygalle et al., 1996). 1-Iodo-4-trimethylsilyloxybutane (**11**) was prepared in situ from iodotrimethylsilane and an excess of tetrahydrofuran and allowed to react under Cu(I) catalysis with the Grignard reagent **10,** which was prepared freshly from the bromide **9.** The TMS ether (**12**) obtained in this way was deprotected with a THF solution of tetrabutylammonium fluoride and acetylated



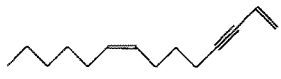
SCHEME 1. (a) EtMgBr/THF; (b) CH_3-CH_2-C≡C-CH_2-OTs (**6**)/Cu(I)Br·Me_2S, −20°C–0°C; (c) Cy_2BH, 4.4 eq.; (d) CH_3CO_2H; (e) PPh_3/Br_2 1.5 eq, CH_2Cl_2; (f) Mg, ether 0–5°C; (g) I-$(CH_2)_4$-OTMS (**11**), CuCN·$(LiCl)_2$, −30°C; (h) tetrabutylammonium fluoride/THF; (i) Ac_2O/pyridine.



SCHEME 2. (a) *n*-BuLi/hexane; (b) CH_3-$(CH_2)_4$-Br, DMPU, 0°C; (c) P-2 Ni/H_2; (d) PPh_3/Br_2 1.5 eq, CH_2Cl_2, 0°C; (e) Li-≡-OTHP, DMPU/THF 0°C; (f) Dowex/MeOH; (g) $LiAlH_4$/diglyme, 120–140°C; (h) Ac_2O/pyridine.

to give the desired dienyl acetate **2** in 34% yield based on the starting bromide **9.**

(3*E*,8*Z*)-3,8-Tetradecadienyl acetate (**3**) was synthesized according to the reaction shown in Scheme 2. This synthesis also employed procedures similar to those used for the synthesis of (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate (**1**), the major pheromone component (Attygalle et al., 1996). An alkylation of the lithiated 1-(tetrahydropyran-2-yloxy)-4-pentyne (**5**) in THF with *n*-pentyl bromide, using 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU) as the base, gave the THP protected alcohol **13.** The reduction of the triple bond in **13** to a *Z* double bond was accomplished by using P2 nickel catalyst (Brown and Ahuja, 1973). The reaction proceeded with high stereoselectivity (98.3% *Z* isomer). Subsequently, using 1.5 eq of $PPh_3 \cdot Br_2$ (Bestmann and Gunawardena, 1992), the tetrahydropyranyl protected hydroxyl group in **14** was replaced by a bromine atom. A coupling of the lithiated 1-(tetrahydropyran-2-yloxy)-3-butyne with the above-prepared bromide **15,** using DMPU as the base, gave the desired THP protected alkeneynol **16.** However, this reaction also produced a nonpolar impurity, which showed a similar R_f value but a different GC retention time compared to those of the starting bromide **15.** This by-product was removed from the bromide **15** by repeated silica gel chromatography. Based on spectral data, this impurity was identified as (*Z*)-1,8-tetradecadien-3-yne (19, Figure 2).



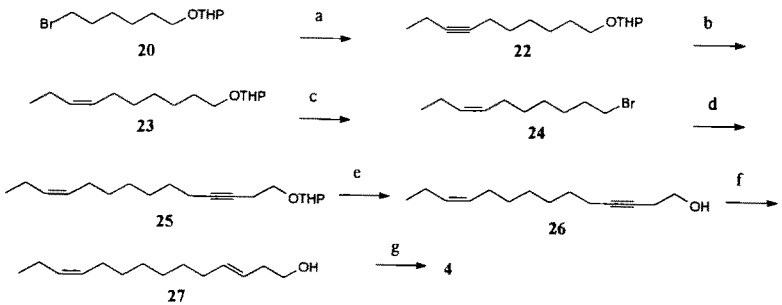
19

FIG. 2. Structure of compound **19.**

This by-product might be expected from a base-catalyzed elimination of the homopropargylic tetrahydropyranyl group. Although this process, to the best of our knowledge, has not been described previously, we have observed other instances in which THP protected 3-alkyn-1-ols give unsaturated impurities even under acidic conditions (TsOH/MeOH also gives rise to the hydrocarbon by-product **19**). In any case, the tetrahydropyranyl protecting group in **16** could be successfully removed, using a Dowex ion-exchange resin in MeOH at room temperature. The product, alcohol **17**, was reduced stereospecifically to dienol **18** with $LiAlH_4$ in diglyme (Rossi and Carpita, 1977). Finally, the reaction mixture containing the dienol **18** was acetylated with Ac_2O/pyridine to give the desired dienyl acetate **3**. Chromatography on a silver-nitrate impregnated silica gel column gave the final product, (3*E*,8*Z*)-3,8-tetradecadien-1-yl acetate **3**, in 97% purity and 17% overall yield (based on lithiated 1-(tetrahydopyran-2-yloxy)-4-pentyne) (Scheme 2).

For the synthesis of (3*E*,11*Z*)-3,11-tetradecadien-1-yl acetate (**4**), we used a procedure similar to that used for the preparation of (3*E*,8*Z*)-3,8-tetradecadien-1-yl acetate (**3**) (Scheme 2). Starting from 1-butynyllithium (**21**) and 1-bromo-6-(tetrahydropyran-2-yloxy)-hexane (**20**), we prepared (3*E*,11*Z*)-3,11-tetradecadien-1-yl acetate in 15% overall yield and in 96% stereoisomeric purity (Scheme 3).

The ion-trap electron-ionization mass spectra of dienes **2**, **3**, and **4** were recorded and compared with those obtained under similar conditions a few months earlier from *S. absoluta* females. Although spectra recorded at different times, even under similar conditions, tend to differ slightly, a comparison of relative intensities within each cluster of *m/z* values provides a more reliable way of asserting whether two spectra represent the same compound or not. An application of this procedure to the spectra presented in Figure 3 disclosed that the



SCHEME 3. (a) Li—≡—/(**21**), DMPU, 0°C; (b) P-2 Ni/H_2; (c) PPh_3/Br_2 1.5 eq, CH_2Cl_2, 0°C; (d) Li—≡—OTHP, DMPU/THF 0°C; (e) Dowex/MeOH; (f) $LiAlH_4$/diglyme, 120–140°C; (g) Ac_2O/pyridine.

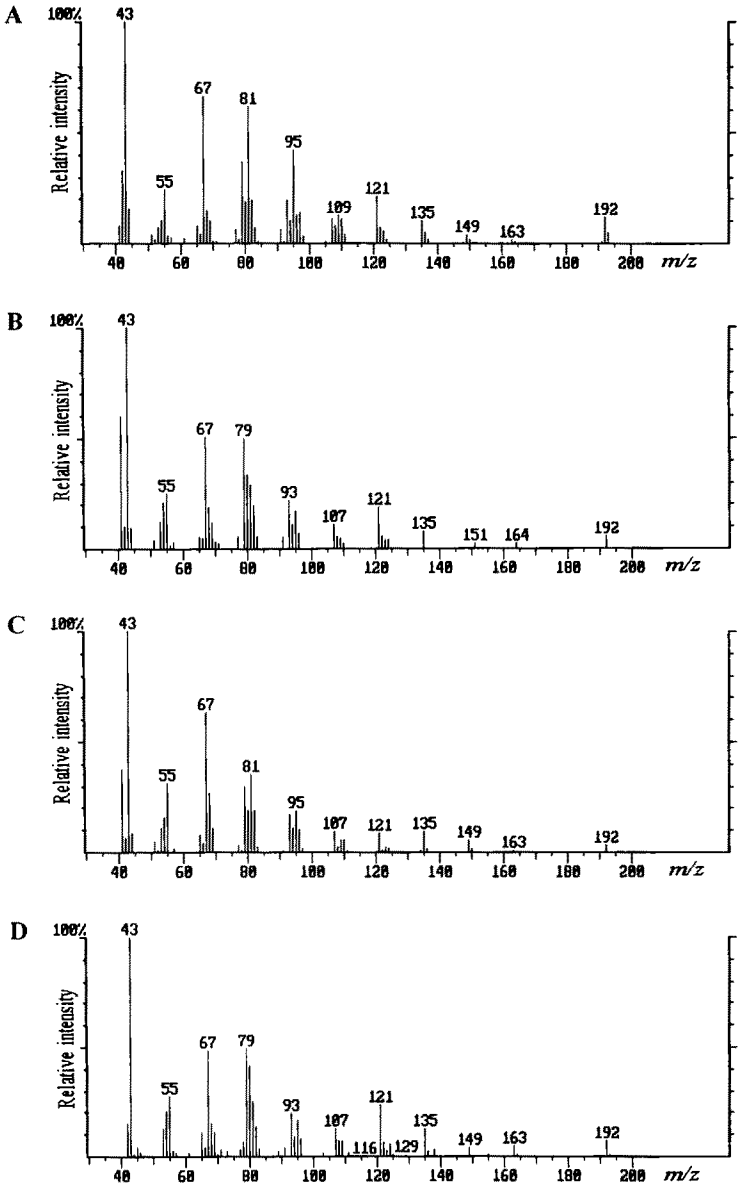


FIG. 3. Ion-trap electron-impact mass spectra of (8*Z*,11*Z*)-8,11-tetradecadien-1-yl acetate (A), (3*E*,8*Z*)-3,8-tetradecadien-1-yl acetate (B), (3*E*,11*Z*)-3,11-tetradecadien-1-yl acetate (C), and the diene acetate from *S. absoluta* (D).

spectrum of synthetic (3*E*,8*Z*)-3,8-tetradecadien-1-yl acetate (**3**) is congruent with that of the natural product. The relative intensities of peaks centered near *m/z* 79 and 91 were particularly useful for characterization of each isomer.

Behavioral activity released by the three synthetic dienes (**2–4**), and by (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate (**1**) was studied in a wind-tunnel bioassay. The diene acetates, although they released some wing-fanning behavior, were essentially inactive, since none of the test insects landed at the source (Figure 4). On the other hand, (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate, the major component of the pheromone, is active by itself, as observed in our previous experiments (Attygalle et al., 1995; 1996). Under the test conditions used in the present study, 53% of test males (N = 30) landed at the source baited only with the triene acetate, while three calling females could induce

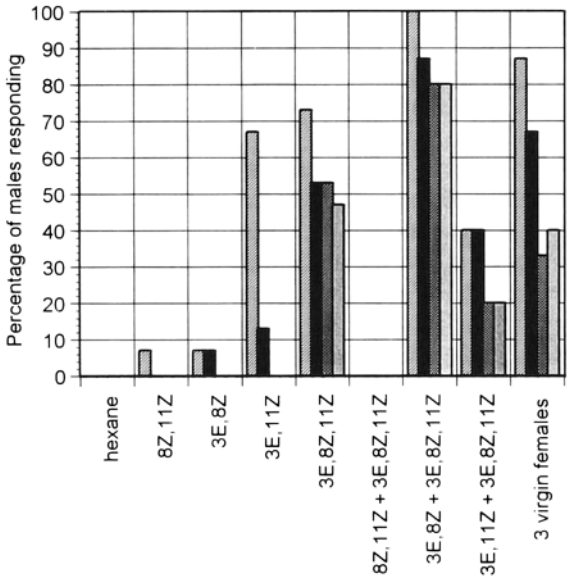


FIG. 4. Percentage of male moths responding in a wind-tunnel behavioral bioassay. Hatched, black, cross-hatched, and stippled bars represent wing fanning, oriented flight, landing at the source, and courtship behavior, respectively. Behavior of 30 males was observed for each test stimulus. Hexane (100 μl) and three calling females were used as control stimuli. Samples of (8*Z*,11*Z*)-, (3*E*,8*Z*)-, and (3*E*,11*Z*)-tetradecadien-1-yl acetate, and (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate were tested individually at the 100-ng level. Binary mixtures contained 10 ng of a diene acetate (**2–4**) and 100 ng of the triene acetate (**1**).

only 33% of the test males to behave similarly. However, the activity released by the binary mixture made of (3*E*,8*Z*)-3,8-tetradecadien-1-yl acetate (**3**) and (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrien-1-yl acetate (**1**) was significantly better (chi-square analysis, $P < 0.001$) than that of the triene acetate alone (Figure 4). This mixture induced 80% of the males to land at the source and show courtship behavior. Interestingly, the activity of the triene acetate is inhibited dramatically by the presence of (8*Z*,11*Z*)-8,11-tetradecadien-1-yl acetate (**2**). The potency of (8*Z*,11*Z*)-8,11-tetradecadien-1-yl acetate as an inhibitor is unequivocal; a binary mixture (10:1 of **1** and **2**) completely failed even to release wing-fanning behavior.

Over 400 compounds have been characterized as constituents of lepidopteran female sex pheromones (Arn et al., 1992; Mayer and McLaughlin, 1991). However, acetates **1** and **3** of *S. absoluta* have not been identified from any other insect. Although our two-component synthetic mixture was more attractive to *S. absoluta* males than the triene acetate alone, it was clear that *S. absoluta* is far less sensitive to the absence of the minor component than most other Lepidoptera that are characteristically highly sensitive to small qualitative or even quantitative changes in the composition of pheromone blends. As we gather more and more data on the composition of lepidopteran pheromones, it will be interesting to see whether moths using structurally less complicated chemicals as pheromones, such as (*Z*)-11-tetradecenyl acetate (a simple ester shared by many species of Lepidoptera as a component of pheromone blends), are more sensitive to qualitative and quantitative variations in composition than are those using less common, more "information-rich" structures.

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EFFECTS OF ALLELOCHEMICALS ON PLANT RESPIRATION AND OXYGEN ISOTOPE FRACTIONATION BY THE ALTERNATIVE OXIDASE

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Abstract—The goal of this investigation was to determine the effects of allelochemicals on plant respiration that thereby may be responsible for their role in growth inhibition. We have tested the effects of juglone, quercetin, cinnamic acid, and α-pinene on respiration rates, and electron partitioning through the cytochrome and alternative respiratory pathways, by measuring on-line oxygen consumption and oxygen isotope fractionation in soybean cotyledon tissue. Cinnamic acid and α-pinene decreased the oxygen consumption rate and increased the relative partitioning of electron transport to the alternative pathway. Possible biochemical mechanisms of these effects are discussed.

Key Words—Allelopathy, alternative oxidase, α-pinene, cinnamic acid, juglone, on-line ^{18}O fractionation, quercetin, respiration, soybean.

INTRODUCTION

There remains considerable controversy as to the ecological relevance of allelopathy, and not all ecologists accept the concept as a significant competitive factor in plant communities (Harper, 1977; Harborne, 1993; Peñuelas, 1993).

We aimed to determine the possible effects of allelochemicals on plant energy metabolism and consequently their role in growth inhibition. We have tested the effect of some typical, widely occurring allelochemicals from different

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chemical families (the quinone juglone, the flavonoid quercetin, the phenolic cinnamic acid, and the terpenoid α-pinene) on soybean cotyledon respiration rates and on partitioning through cytochrome and alternative respiratory pathways. We studied the respiratory response of soybean cotyledons, these being one of the most widely used vegetable materials in plant respiration studies. We avoided problems associated with the traditional use of inhibitors such as SHAM or KCN by using on-line fractionation against ^{18}O, which is substantially greater by the alternative oxidase than by cytochrome oxidase (Guy et al., 1989; Robinson et al., 1992).

METHODS AND MATERIALS

We conducted on-line measurements of oxygen consumption and oxygen isotope fractionation (Δ) during dark respiration by soybean cotyledons. Immediately before recording the measurements, 30 soybean cotyledons in darkness were bathed for 30 min in $CaCl_2$ aqueous solutions with 10 mM of each of the tested allelochemicals (quercetin, juglone, and cinnamic acid from Sigma Chemical Co.) except for α-pinene (also from Sigma Chemical Co.), which was kept in an open beaker volatilizing inside the gas-tight syringe chamber we used to measure respiration.

Full details of the equipment and methods for these on-line measurements are described in Robinson et al. (1992). In these experiments we introduced the following modifications and improvements. Thirty soybean cotyledons inside a gas-tight 50-ml chamber were allowed to respire. At regular time intervals, gas samples of 100 μl were withdrawn and injected directly into helium flow to a GC-MS, after stripping water and CO_2. For all the measurements the room temperature was 25°C. The fractionation factor (D) was calculated as the slope of the linear regression of $\ln(R/R_0)$ versus $-\ln f$ through the origin (Guy et al., 1989), where f is the fraction of the original O_2 in the reaction chamber, and R_0 and R are the isotope ratios ($^{18}O/^{16}O$) of the initial and subsequent samples (for all the measured relationships r^2 was larger than 0.99). The fractionation Δ was calculated from D using the equation $\Delta = D/(1 - D)$. These experiments consisted of 8–12 oxygen measurements and were replicated three to six times for each of the allelochemicals studied.

RESULTS AND DISCUSSION

Cinnamic acid and α-pinene (the smaller molecules tested, and therefore the ones more likely to penetrate live tissues) decreased the oxygen consumption rate, while quercetin and juglone had no significant effect (Table 1). These results agree with data suggesting perturbations of mitochondrial and chloroplast

TABLE 1. OXYGEN CONSUMPTION RATES, OXYGEN ISOTOPE DISCRIMINATION Δ, AND PARTITIONING OF RESPIRATORY PATHWAYS IN SOYBEAN COTYLEDONS[a]

	Control	Quercetin	Juglone	α-Pinene	Cinnamic
Respiration (μmol O_2 uptake/g dw/hr)	88.63 ± 6.65	112.68 ± 13.3	85.92 ± 15.96	62.91 ± 3.99[b]	57.59 ± 2.66[b]
Discrimination against ^{18}O (‰)	24.3 ± 0.12	23.7 ± 0.16	23.4 ± 0.15	25.2 ± 0.25[b]	26.8 ± 0.36[b]
Alternative pathway flux (μmol O_2 uptake/g dw/hr)	37.74 ± 2.7	34.93 ± 4.3	28.78 ± 4.7	29.81 ± 1.9	35.53 ± 2.3
Cytochrome pathway flux (μmol O_2 uptake/g dw/hr)	50.89 ± 4.2	77.75 ± 9.2	57.14 ± 8.2	33.1 ± 2.4[b]	22.06 ± 1.1[b]

[a]Cotyledons were bathed for 30 minutes (in darkness) in $CaCl_2$ aqueous solutions with 10 mM of each one of the allelochemicals tested, except α-pinene, which was kept in an open beaker volatilizing inside the gas-tight chamber where we measured respiration. Data of three to six experiments per treatment (8–12 oxygen measurements were conducted in each experiment).

[b]Significantly different from the control at 0.05 probability level according to Student's *t* test for two paired groups.

functions linked to plant growth reductions observed in classical allelopathy experiments (Pellesier, 1993; Hejl et al., 1993; see references in Einhellig, 1995). However, they do not agree with the general hierarchy of allelopathic activity: quinones > flavonoids > phenolic acids (Einhellig, 1995).

The fractionation (Δ) ranged between 31‰ when KCN was used to inhibit the cytochrome pathway and 20‰ when SHAM was used to inhibit alternative oxidase. The Δ of 24.3‰ in control treatments was increased to 26.8‰ by cinnamic acid treatment (Figure 1), indicating an increased percentage of respiratory flux to the alternative pathway from 39 to 62%. The relative partitioning of electron transport to the alternative oxidase pathway was also increased (higher Δ) by α-pinene (Table 1), but was not significantly changed by quercetin or juglone (Table 1). However, none of these allelochemicals changed the absolute electron flux through the alternative pathway (Table 1). Thus, the inhibition of respiration occurred in the cytochrome pathway and possibly in glycolysis; otherwise the redox state of the ubiquinone pool would have increased and enhanced the flux through alternative pathway. The effects on respiration could be secondary through other cell functions, such as, for example, ion uptake, which are energy demanding. Further work on isolated mitochondria is necessary to clarify the situation.

The studies conducted here dealt with responses of isolated allelochemicals,

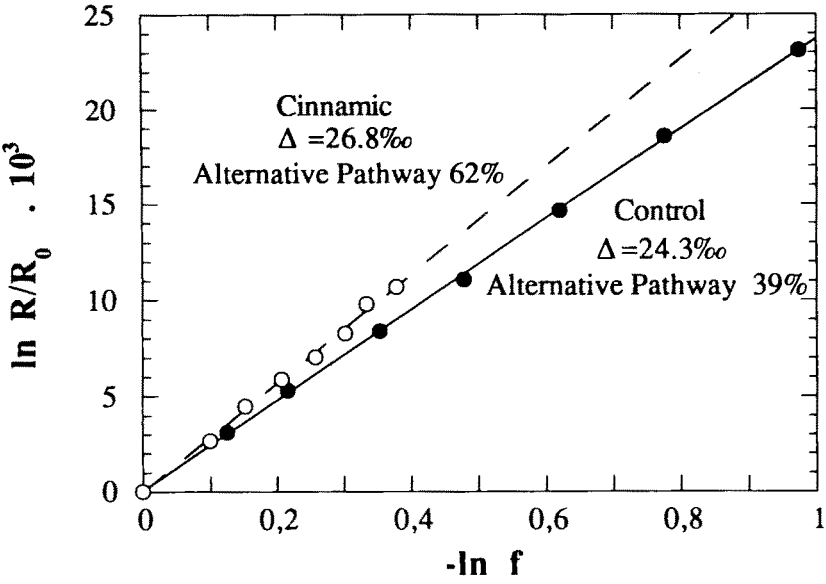

FIG. 1. Example of on-line measurements of oxygen consumption and oxygen isotope fractionation, Δ, during dark respiration by soybean cotyledons. The fraction of the original O_2 in the reaction chamber is f, and R_0 and R are the isotope ratios ($^{18}O/^{16}O$) of the initial and subsequent samples ($r^2 > 0.99$ for all the measured relationships between $\ln R/R_0$ and $-\ln f$). The Δ ranged between 31‰ when KNC was used to inhibit the cytochrome pathway and 20‰ when SHAM was used to inhibit alternative oxidase. Therefore, partitioning to the alternative pathway was calculated as $(\Delta - 20)/(31 - 20)$. These measurements were replicated three to six times for each of the allelochemicals studied (results in Table 1).

and in the short-term, of the order of 1 hr. Of course, such an approach is far from the extreme chemical complexity of the soil and air environment, and additional studies are required to determine how and at what concentrations these substances become actual allelochemicals. Nevertheless, the method used here provides an indication that these allelochemicals alter normal oxygen uptake and may influence significant competitive effects on neighboring plants.

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ATTRACTION OF *Ceratitis capitata* (DIPTERA: TEPHRITIDAE) FLIES TO ODOR OF COFFEE FRUIT

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Abstract—On potted nonfruiting host trees in outdoor field cages, we evaluated attraction of released mature laboratory-cultured or wild-origin Mediterranean fruit flies (medflies) to odor of freshly picked fruit of host and nonhost plants. Odor of ripe intact or crushed coffee fruit (the presumed ancestral host of medflies) was significantly more attractive than odor of ripe intact or crushed fruit of five lower-ranking hosts and three nonhosts. Odor of crushed coffee fruit was significantly more attractive than odor of intact coffee fruit. Odor of ripe or near-ripe coffee fruit was significantly more attractive than odor of unripe coffee fruit. Immature females (without eggs) were significantly more attracted to odor of a proteinaceous food lure than to odor of ripe coffee fruit, whereas the reverse was true for mature females carrying a high egg load. In some trials, males proved as discriminating as females in favor of coffee fruit odor, but in several other trials males were less discriminating than females. Response patterns of mature laboratory-cultured females were similar to those of mature wild-origin females. In a field of coffee plants, attraction of natural-population females was significantly greater to odor of ripe coffee fruit than to water but was not greater than attraction to odor of proteinaceous food. Findings are discussed in relation to potential use of synthetic volatiles of coffee or other host fruit in traps for monitoring or controlling medflies.

Key Words—Attractants, odor, host fruit, coffee fruit, *Ceratitis capitata*.

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INTRODUCTION

Numerous kinds of insects are known to be attracted to odor of their host plants. Most examples involve monophagous or oligophagous species, but in a few cases, polyphagous insects also have exhibited attraction to host plant odor (examples given in Bernays and Chapman, 1994). In tephritid flies, adult attraction to the odor of host fruit in favorable condition for oviposition has been demonstrated in the monophagous papaya fruit fly, *Toxotrypana curvicauda* Gerstaecker (Landolt et al., 1992), the monophagous olive fruit fly, *Bactrocera oleae* (Gmelin) (Scarpati et al., 1993), the oligophagous apple maggot fly, *Rhagoletis pomonella* (Walsh) (Prokopy et al., 1973; Averill et al., 1988), the polyphagous oriental fruit fly, *Bactrocera dorsalis* Hendel (Jang and Light, 1991), the polyphagous Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Eisemann and Rice, 1992), and the polyphagous Caribbean fruit fly, *Anastrepha suspensa* (Loew) (Nigg et al., 1994).

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann), is a highly polyphagous tephritid that oviposits and develops in more than 300 species of fruits and vegetables (Liquido et al., 1991). To date most studies of medfly reaction to plant odor have involved measuring electroantennogram responses to a spectrum of plant volatiles, including several volatile constituents of host fruit and foliage (e.g., Guerin et al., 1983; Light et al., 1988, 1992). Regarding behavioral responses of medflies to plant odor, Levinson et al. (1990) presented laboratory-cage evidence suggesting lack of medfly attraction to odor of oranges, *Citrus sinesis* (a principal host), but in field cage tests, Katsoyannos et al. (1995) have shown that both sexes of medflies are in fact attracted to odor of oranges. Using an outdoor olfactometer, Keiser et al. (1975) demonstrated significantly greater captures of medflies in traps baited with solvent extracts of various structures of a wide variety of plants (nearly all of which were nonhosts) compared with captures in unbaited traps. Finally, under field conditions, Guerin et al. (1983) found that medflies were attracted to traps baited with heptanal, a constituent of the fruit of several medfly host plants.

Here, in tests conducted in a patch of nonfruiting host trees in an outdoor field cage, we asked whether medflies were more attracted to odor of the fruit of coffee, *Coffea arabica* (the presumed ancestral host of medflies in Africa (Vargas et al., 1995)), than to odor of lower-ranking host fruit and nonhost fruit. We also asked whether both sexes were equal in discrimination among fruit odors of the various types tested, whether crushed fruit was more attractive than intact fruit, whether ripe fruit was more attractive than unripe fruit, whether attraction to host fruit odor was greater or lesser than that to food odor among flies of differing physiological states, and whether response patterns of wild-origin medflies differed from those of laboratory-cultured medflies. Finally, we

asked whether natural-population medflies in the field exhibited a level of response to host fruit odor similar to that of medflies in the field cage.

METHODS AND MATERIALS

Medflies used in field cage tests originated either from a laboratory colony in culture for 10 generations at the USDA Tropical Fruit and Vegetable Research Laboratory in Honolulu or from natural-population larvae that infested field-collected coffee fruit. Unless indicated otherwise, from eclosion until tested 7–10 days afterward (for lab-cultured flies) or 14–18 days afterward (for wild-origin flies), both sexes were held together in 30- × 30- × 30-cm screened cages supplied with protein (enzymatic yeast hydrolysate), sucrose, and water but not fruit under laboratory conditions of about 25°C, 50% relative humidity, and 13 hr natural daylength.

Besides coffee, fruit used in field cage trials included five species that we considered to be low-ranking host fruit because in a 36-year survey in Hawaii (Liquido et al., 1990), only 1–9% of collected batches proved to be infested by medfly larvae (compared with 65% of collected coffee fruit batches found to be infested). These low-ranking host fruit included: guava, *Psidium guajava*; banana, *Musca paradisiaca*; tangerine, *Citrus reticulata*; papaya, *Carica papaya*; and avocado, *Persea americana*. We also evaluated three species of fruit that we considered to be nonhosts of medfly because none of the batches collected by Liquido et al. (1990) were found to be infested by medfly. These included: squash, *Cucurbita maxima*; macadamia nut, *Macadamia integrifolia*; and lipstick plant, *Bixa orellana*. All fruit were picked fresh from fruiting plants and placed immediately at 3°C until testing, which occurred 4–96 hr after picking. Unless stated otherwise, only fruit that we considered to be ripe and in optimum condition for potential medfly oviposition were picked and tested. Two hours before testing, fruit were rinsed with water, allowed to dry, and warmed to 25°C.

The first series of field cage experiments involved assessing attractiveness of odor of intact fruit of each type. In these and all other experiments, fruit of a single type was placed in a cylindrical clear-plastic container (11 × 13 cm) whose side and bottom was covered with aluminum foil to obscure visibility of fruit within. All but the 1-cm periphery of the clear-plastic lid consisted of nylon netting that effectively obscured visibility of fruit but permitted air flow. The number of fruit placed in each container was adjusted so that a total of ~ 140 cm^2 of fruit surface area was exposed to air. With coffee, this was equivalent to 30 fruit that filled to capacity the floor of the container. In cases where the surface area of a single intact fruit exceeded ~ 140 cm^2, excess area was tightly

enveloped in aluminum foil to prevent odor escape. The second series of field cage experiments involved assessing attractiveness of the odor of 5 g per container of freshly crushed skin and flesh of fruit of each type. Succeeding field cage experiments involved primarily the evaluation of medfly response to the odor of various conditions of coffee fruit or Nulure (Miller Chemical Co., Hanover, Pennsylvania), a proteinaceous food-type attractant. We formulated Nulure according to Wakabayashi and Cunningham (1991): 9% Nulure, 5% sodium borate, and 86% water. We used an amount that covered the floor of the container.

All field-cage trials were carried out from 09:00–16:00 hr in a cylindrical, 3-m-tall × 3-m-diam., clear nylon-screen enclosure placed outdoors on the grounds of the USDA Tropical Fruit and Vegetable Research Laboratory in Honolulu. The top of the enclosure was covered with a partly opaque tarpaulin to exclude direct sunlight and rainfall. Flow of wind through the cage was regulated to 0–3 km/hr by using an adjustable, clear-plastic wind barrier at the upwind side of the cage wall. The cage contained four potted nonfruiting guava trees grouped near the center of the cage to form a continuous canopy (120 cm tall × 220 cm diam.). Before use, the foliage and stems of each tree were rinsed thoroughly with water.

At each of four positions within the canopy, we hung a single container of test substance. The positions were about 90 cm apart along the circumference of an imaginary circle about 20 cm inward from the periphery of the upper part of the canopy. For each replicate of each experiment, about 25 flies of each sex were released as a group at the lower center of the canopy. We assumed (but did not document) a 50:50 sex ratio of released flies. Flies that arrived on the screened area of the lid of a container within the 5-min period allotted each replicate were considered as responding to odor of the contents, were sexed, and were removed by an aspirator. Treatment positions were randomized for the first replicate. Treatments were rotated clockwise within the canopy after each replicate until each treatment occupied each position once, requiring a total of about 25 min to complete a set of four replicates. For the next set of four replicates, we used a fresh batch of fruit. Most nonresponding flies had flown to the cage wall by the end of a replicate. To shorten time and hence minimize changes in the state of fruit over the course of testing, we adopted a 5-min test period rather than a longer test period and we did not collect and remove nonresponding flies until the end of an experiment. A separate batch of flies was used for each experiment. During experiments, temperature within the tree canopy ranged from 24 to 31°C.

The field test was conducted in a plantation of coffee plants on Kauai (Vargas et al., 1995) which, at the time of testing in March 1995, had recently been harvested. Very few fruit remained. A container of test substance was

inserted into a hole in the center of a 25- × 25-cm piece of horizontal brown cardboard in such a way that the screened lid of the container protruded about 3 cm above the Tangletrap-coated upper surface of the cardboard. Such traps were suspended 10 m apart from the branches of coffee plants. After 1 hr, captured natural-population medflies were counted and removed, the test fruit was replaced with freshly prepared batches, and the containers were moved to new positions.

Data were analyzed by two-way analysis of variance (ANOVA). Treatment means were compared using the least significant difference test criterion (0.05 level). For ANOVA, the 16 replicates per treatment per experiment were grouped into four sets of four replicates each. For each treatment, the value of a single set of four replicates consisted of the number of flies arriving on the screen of a container for all four positions of that treatment combined. We reasoned that grouping the data into sets of replicates for ANOVA was the most valid approach to minimizing varying effects of treatment position within the tree canopy on fly response to replicates within a set.

RESULTS

Field Cage Tests. In four experiments involving intact fruits, the odor of ripe coffee fruit was significantly more attractive to mature (egg-bearing) laboratory-cultured females than was the odor of five kinds of ripe low-ranking host fruit (guava, tangerine, papaya, banana, and avocado) or the odor of three kinds of ripe nonhost fruit (squash, macadamia nut, and lipstick plant) (Table 1). Except for papaya and avocado, the odor of low-ranking hosts was more attractive than water. Except for macadamia nut, the odor of nonhosts was not more attractive than water. In these four experiments, significantly more males were attracted to coffee odor than to the odor of tangerine, papaya, banana, or avocado (but not guava) (Table 1). Among low-ranking hosts, only the odor of banana attracted significantly more males than water. Except for lipstick plant, the odor of nonhosts was no more attractive to males than was water.

In four similarly designed experiments that involved comparing the response of mature laboratory-cultured medflies to the odor of crushed ripe fruit, again significantly more females were attracted to coffee odor than to the odor of any low-ranking host fruit or nonhost fruit (Table 1). Again, except for papaya and avocado, the odor of low-ranking hosts was more attractive than water, and, except for macadamia nut, the odor of nonhosts was no more attractive than water. In these four experiments, significantly more males were attracted to coffee odor than to the odor of papaya and avocado (but not guava, banana, or tangerine) (Table 1). Among low-ranking hosts, the odor of banana and tangerine (but not papaya, guava and avocado) attracted significantly more males

TABLE 1. ATTRACTION OF MATURE RELEASED LABORATORY-CULTURED *C. capitata* FLIES TO ODOR OF INTACT OR CRUSHED RIPE HOST OR NONHOST FRUIT IN CONTAINERS IN NONFRUITING FIELD-CAGED TREES

Experiment	Fruit condition	Fruit type	Arriving flies (mean *N*) per replicate[a]	
			Females	Males
1	Intact	Coffee	5.7a	1.1a
		Guava	2.6b	0.6ab
		Squash	1.9bc	0.6ab
		Water	1.3c	0.1b
2	Intact	Coffee	6.9a	1.3a
		Tangerine	2.0b	0.4b
		Papaya	1.6bc	0.3b
		Water	0.3c	0.4b
3	Intact	Coffee	8.3a	3.6a
		Banana	4.3b	1.6b
		Mac Nut	1.9c	1.1bc
		Water	0.3d	0.5c
4	Intact	Coffee	8.6a	3.3a
		Avocado	1.9b	0.6bc
		Lipstick	2.6b	2.3ab
		Water	1.8b	1.1c
5	Crushed	Coffee	5.9a	3.3a
		Guava	3.3b	2.0ab
		Squash	1.4c	1.3ab
		Water	1.1c	0.8b
6	Crushed	Coffee	6.3a	3.1a
		Tangerine	3.3b	2.8a
		Papaya	1.9bc	1.1b
		Water	0.4c	0.6b
7	Crushed	Coffee	10.2a	4.8a
		Banana	5.0b	4.4a
		Mac Nut	4.4b	2.1b
		Water	1.4c	1.1b
8	Crushed	Coffee	9.0a	2.1a
		Avocado	1.4b	0.5b
		Lipstick	2.0b	2.5a
		Water	0.9b	0.6b

[a]Sixteen replicates per treatment. Values in each column in each experiment followed by the same letter are not significantly different at the 0.05 level according to least significant difference tests.

than water. Except for lipstick plant, the odor of nonhosts was no more attractive to males than water.

Each sex of mature laboratory-cultured medflies was significantly more attracted to the odor of 30 ripe crushed coffee fruit than to the odor of 30 ripe intact coffee fruit (Table 2). The odor of 30 ripe (dark red) and 30 near-ripe (orange-red) intact coffee fruit was no different in attractiveness to either sex of laboratory-cultured medfly; each was significantly more attractive to each sex than the odor of 30 unripe (green) intact coffee fruit, which proved to be no more attractive than water (Table 2).

One-day-old, laboratory-cultured, protein-fed females were significantly more attracted to the odor of Nulure than to the odor of 30 intact ripe coffee fruit, which was no more attractive than water or an empty container (Table 3). Three-day-old, laboratory-cultured, protein-fed females (having immature eggs only) responded similarly to 1-day-old females, except that coffee odor was now significantly more attractive than water or an empty container. In contrast, 10-day-old, laboratory-cultured, protein-fed females (having a large complement of mature eggs) were significantly more attracted to coffee odor than to Nulure, which was no more attractive than water or an empty container. Ten-day-old, laboratory-cultured, protein-deprived females (that had received only sucrose since eclosion) responded similarly to 3-day-old protein-fed females. The response pattern of 1-day-old and 10-day-old protein-fed males was the same as that of equivalent-type females, but 3-day-old protein-fed males and 10-day-old protein-deprived males did not discriminate among treatments (Table 3).

TABLE 2. ATTRACTION OF MATURE RELEASED LABORATORY-CULTURED *C. capitata* FLIES TO ODOR OF INTACT OR CRUSHED RIPE COFFEE FRUIT AND ODOR OF COFFEE FRUIT IN THREE STAGES OF RIPENESS IN CONTAINERS IN NONFRUITING FIELD-CAGED TREES

Experiment	Fruit condition	Arriving flies (mean *N*) per replicate[a]	
		Females	Males
1	Intact	1.8b	1.6b
	Crushed	4.6a	4.4a
2	Green	3.5b	1.7b
	Orange-red	9.0a	4.1a
	Dark red	10.9a	5.3a
	Water	1.5b	0.6b

[a] See footnote of Table 1.

TABLE 3. ATTRACTION OF RELEASED LABORATORY-CULTURED *C. capitata* FLIES OF DIFFERENT AGES OR PROTEIN-ACCESS TREATMENTS TO ODOR OF INTACT RIPE COFFEE FRUIT OR PROTEINACEOUS FOOD (NULURE) IN CONTAINERS IN NON-FRUITING FIELD-CAGED TREES

Experiment	Condition of flies	Type of attractant	Arriving flies (mean *N*) per replicate[a]	
			Females	Males
1	One day old, protein fed	Coffee	0.8b	0.4b
		Nulure	2.8a	1.4a
		Water	0.3b	0.2b
		None	0.1b	0.0b
2	Three days old, protein fed	Coffee	1.3b	0.4a
		Nulure	3.0a	0.3a
		Water	0.4c	0.1a
		None	0.4c	0.0a
3	Ten days old, protein fed	Coffee	2.8a	1.6a
		Nulure	0.6b	0.4b
		Water	0.6b	0.6b
		None	0.6b	0.5b
4	Ten days old, protein deprived	Coffee	2.1b	0.0a
		Nulure	3.1a	0.5a
		Water	0.3c	0.1a
		None	0.1c	0.1a

[a] See footnote of Table 1.

TABLE 4. ATTRACTION OF MATURE RELEASED LABORATORY-CULTURED OR WILD *C. capitata* FLIES TO ODOR OF INTACT RIPE COFFEE OR GUAVA FRUIT OR PROTEINACEOUS FOOD (NULURE) IN CONTAINERS IN NONFRUITING FIELD-CAGED TREES

Experiment	Type of flies	Type of attractant	Arriving flies (mean *N*) per replicate[a]	
			Females	Males
1	Lab-cultured	Coffee	2.4a	3.2a
		Guava	0.9b	1.1b
		Nulure	0.6b	1.1b
		Water	0.6b	0.6b
2	Wild	Coffee	2.6a	0.3a
		Guava	1.1b	0.3a
		Nulure	1.3b	0.8a
		Water	0.4b	0.1a

[a] See footnote of Table 1.

TABLE 5. ATTRACTION OF NATURAL-POPULATION *C. capitata* FLIES TO ODOR OF CRUSHED RIPE COFFEE FRUIT OR PROTEINACEOUS FOOD (NULURE) IN CONTAINERS IN A FIELD OF NONFRUITING COFFEE PLANTS

Type of attractant	Arriving flies (mean *N*) per treatment[a]	
	Females	Males
Coffee	1.6a	0.2b
Nulure	2.3a	2.7a
Water	0.2b	0.6b

[a] Same as footnote of Table 1 except 12 replicates per treatment.

When compared for attraction to the odor of equivalent surface areas of intact coffee fruit, intact guava fruit, Nulure, and water, mature, protein-fed, wild-origin and laboratory-cultured females responded similarly: they showed significantly greater attraction to coffee odor than to the odor of the other three treatments, among which there were no significant differences (Table 4). The same pattern held true for laboratory-cultured males but not for wild-origin males, which did not discriminate among treatment types (Table 4).

Field Test. Natural-population females in a coffee plantation were significantly more attracted to the odor of Nulure and 30 near-ripe crushed coffee fruit (no significant difference between these) than water (Table 5). Natural-population males were significantly more attracted to the odor of Nulure than to the odor of coffee fruit, which was no more attractive than water (Table 5).

DISCUSSION

Our findings show that mature laboratory-cultured medfly females were attracted to the odor of near-ripe and ripe coffee fruit (particularly recently crushed fruit), but not to the odor of unripe coffee fruit. Female response was weaker to the odor of tested types of lower-ranking host fruit and generally lacking to the odor of tested types of nonhost fruit, irrespective of whether such fruit were intact or crushed. Laboratory-cultured females carrying no mature eggs proved to be more attracted to the odor of proteinaceous food (Nulure) than to the odor of ripe intact coffee fruit, but the reverse was true for mature, egg-bearing females. When directly compared, response patterns of mature wild-origin females proved similar to those of mature laboratory-cultured females. Natural-population females were found to be attracted to odor of both Nulure and coffee fruit.

This is the first published study to show that the odor of fresh-picked coffee

fruit, the presumed ancestral host of medflies, is more attractive to medflies than fruit odor of lower ranking host and nonhost plants. To our knowledge, there exist three other studies that have examined the potential attractiveness of coffee plant odor to medflies. First, Keiser et al. (1975) found significant attraction of medfly females and males to ether extracts of coffee bark and stems, but they did not make extracts of coffee fruit. Such extracts proved more attractive than extracts of foliage, bark, or stems of most nonhosts tested, but odor of some nonhosts (e.g., *Diospyros discolor*, *Kallstroemia maxima*) proved more attractive than coffee odor. Second, Vargas and Chang (1991) found that harvest of eggs from oviposition bottles that contained a hot water extract of canned coffee grounds was significantly greater than that from bottles that contained orange, guava, or papaya juice. Third, Lance, Jang, and McInnis (unpublished data) have recent evidence that somewhat parallels the findings of our study: crushed ripe coffee fruit is attractive to both sexes of medflies under laboratory wind-tunnel as well as field conditions. Further research is needed to determine whether the odor of coffee fruit is more or less attractive than the odor of coffee foliage, stems, or woody tissue.

Our finding that the odor of near-ripe and ripe coffee fruit is much more attractive to medflies than the odor of unripe coffee fruit is consistent with the finding of Vargas et al. (1995) that oviposition by natural-population medflies in commercial plantations was dramatically (30 to 70-fold) greater in ripe than unripe coffee fruit. Similarly, Carle et al. (1987) showed that the odor of host fruit of *R. pomonella* was much more attractive to the flies when fruit was ripe than unripe. Furthermore, Jang and Light (1991) found that odor of host fruit of *B. dorsalis* increased in attractiveness to the flies with increasing ripeness of fruit. Together, these findings suggest that future study of the identity of volatile components of coffee plants attractive to medflies should be focused on plants whose fruit are near-ripe or ripe.

We were not surprised that the odor of freshly crushed coffee fruit was more attractive to medflies than the odor of intact coffee fruit. First, one would expect that a greater amount of volatile chemicals is released from recently wounded plant tissue than intact plant tissue (Finch, 1980). Second, Papaj et al. (1989, 1992) demonstrated that medfly females in search of egg-laying sites can respond positively and quickly to recent natural or artificial punctures in host fruit. Exploitation of fresh punctures as potential oviposition sites may be adaptive when females are limited in time available to drill punctures in fruit or when fruit are resistant to puncturing (Papaj et al., 1992), particularly in habitats where predators of ovipositing females are abundant (Papaj et al., 1989). Selection may have given rise to a high level of medfly sensitivity to the odor of host fruit released from fresh punctures (magnified here in the form of freshly crushed host fruit).

Our finding that female medflies were in several instances more responsive to coffee fruit odor than were male medflies parallels the finding of Keiser et al. (1975) that females were somewhat more attracted than males to the odor of coffee stems and bark. In addition, both Levinson et al. (1990) and Light et al. (1992) describe several instances of greater antennal receptor sensitivity of female than male medflies to host fruit volatiles. The biological basis of more frequently detected positive responses of medfly females than males to host odor is uncertain, but it could reside in a strong influence upon females of host odor as indicative of host plants or individual host fruit that are potentially favorable egg-laying sites, whereas host odor might have only a weak influence upon males as indicative of potentially favorable mating sites. Mating in medflies apparently occurs in the vicinity of host plants but is not always restricted to host plants (Hendrichs and Hendrichs, 1990).

The physiological state of an insect and the structure of the resource environment may have important effects on the order in which an insect prioritizes its visits to essential types of resources, such as feeding and egg-laying sites. Precisely how hunger and egg load interact in shaping resource foraging behavior has received considerable attention in insects such as *Blatella* cockroaches, *Phormia* and *Lucilia* blowflies, *Musca* face flies, *Glossina* tsetse flies, and several species of mosqitoes that have distinct ovarian cycles in which response to food stimuli and feeding gradually decline as a developing batch of oocytes approaches maturity (reviewed in Barton Browne, 1993). In tephritid flies, there is strong evidence indicating that *B. tryoni* and *R. pomonella* females denied access to protein and lacking fully developed eggs are more attracted to proteinaceous food than to egg-laying sites, whereas the opposite is true for females provided with protein and carrying moderate to large egg loads (Prokopy et al., 1991, 1995). Our findings here with laboratory-caged medflies whose age and diet prior to testing in field cages were carefully defined are consistent with the pattern exhibited by *B. tryoni* and *R. pomonella*. Unfortunately, we had no knowledge of fly age or feeding history in our study of natural-population medflies in the coffee field. We can only speculate that for whatever reason, those captured were roughly equally divided between females seeking proteinaceous food and females seeking egg-laying sites.

Together, the results of this study have implications for the design of traps used to monitor or control medflies. For example, current traps for medfly females rely solely upon proteinaceous food-type stimuli such as Nulure as attractants (Millar, 1995). If the environment in which food-type traps are placed has abundant natural food, such as honeydew or bird droppings (Nishida, 1980; Prokopy et al., 1992), or if females are not hungry for protein, then food-type traps may be comparatively ineffective in luring medflies. Synthetic host fruit odor could be a valuable complement to food odor in that it could attract females

seeking oviposition sites rather than food. Traps baited with synthetic fruit odor alone have proven very effective in monitoring *R. pomonella* flies in apple orchards (Agnello et al., 1990), while traps baited with a combination of synthetic food and synthetic fruit odor have provided good to excellent commercial control of *R. pomonella* (Prokopy et al., 1994). As pointed out by Millar (1995), competition from fruit odor emitted by host plants themselves and prior experience of natural-population females would have to be considered when developing strategies for deploying medfly traps baited with synthetic fruit odor. Before an intensive effort is made to identify and synthesize host volatiles attractive to female medflies, however, further study is needed to pinpoint which coffee plant structures and which phenological stages of coffee plant development do in fact emit the most attractive odor. Moreover, further study is needed to determine if the odor of coffee plants is more attractive than the odor of other high-ranking host plants of medfly. Finally, research is needed to assess whether a combination of host fruit odor and proteinaceous food odor is more attractive to natural-population medflies than either type of odor alone.

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BEHAVIORAL RESPONSE OF FEMALE *Helicoverpa (Heliothis) armigera* HB. (LEPIDOPTERA: NOCTUIDAE) MOTHS TO SYNTHETIC PIGEONPEA (*Cajanus cajan* L.) KAIROMONE

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Abstract—Female *H. armigera* moths are highly attracted by a steam distillate from pigeonpea plants, one of their main hosts. A mixture of six compounds, all sesquiterpenes (β-caryophyllene, α-humulene, α-guajene, α-muurolene, γ-muurolene, and α-bulnesene), mixed in the proportions as found in the steam distillate, elicited the same behavioral responses (oriented upwind flights and contacts with the odor source) as the steam distillate. Only α-bulnesene was attractive by itself, but still less than the whole mixture. In addition, the sesquiterpene mixture acts as an oviposition stimulant. Both behavioral responses, orientation and oviposition, are concentration dependent. Electrophysiological recordings from female and male antennae (EAG) showed the same qualitative and quantitative responses to each of the compounds of the sesquiterpene mixture. The EAG responses to the original steam distillate were higher and similar to chickpea kairomonal components, which were also tested. The pigeonpea sesquiterpene mixture and its individual components elicited weak EAG responses only. The response of the male antenna to female-produced pheromone components was in the same range as the pigeonpea steam distillate.

Key Words—Attractant, *Cajanus cajan*, EAG, *Helicoverpa armigera*, kairomone, Lepidoptera, Noctuidae, oviposition stimulant, pheromone, pigeonpea, sesquiterpene, β-caryophyllene, α-humulene, α-guajene, α-muurolene, γ-muurolene, α-bulnesene.

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INTRODUCTION

Chemical stimuli from plants play an important role in insect–plant interactions. In the order Lepidoptera, to which some of the most harmful noctuid agricultural pests belong, much work has been done on the effect of plant volatiles that act as ovipositional stimulants or deterrents (Renwick, 1989; Renwick and Chew, 1994). Little is known, however, about long-range perceptible host-plant signals. Use and manipulation of semiochemicals are important components of an integrated pest management strategy (Rembold and Schroth, 1993).

The noctuid podborer, *Helicoverpa armigera* (Hb.) (Lepidoptera: Noctuidae), is highly polyphagous, like its near relatives, *H. zea*, *H. punctigera*, and *Heliothis virescens*. For two species, *H. virescens* and *H. zea*, it was demonstrated that extracts from their host plants were ovipositional stimulants (Jackson et al., 1986; Mitchell and Heath, 1987; Mitchell et al., 1990; Tingle et al., 1990). *H. armigera* females are significantly attracted by a mixture of four compounds identified in the headspace of chickpea (*Cicer arietinum*) seed powder, three of which are monoterpenes (Rembold et al., 1991). Carbon dioxide has recently been identified as another potent semiochemical for *H. armigera* larvae (Rasch and Rembold, 1994).

Another economically important host plant of *H. armigera* is the pigeonpea, *Cajanus cajan*. Individual eggs are laid scattered on buds, flowers, and leaves. Preliminary studies with headspace volatiles emanating from the seedlings of two pigeonpea varieties of different susceptibility to *H. armigera* attack demonstrated a clear preference of the ovipositing moth for the more susceptible pigeonpea variety; the volatile attractants were identified in the hexane soluble fraction containing mono- and sesquiterpenoids (Rembold and Tober, 1985). We found by application of an olfactometer bioassay (Rembold et al., 1989) that the pigeonpea steam distillate, as well as a synthetic mixture of six pure compounds, were highly attractive to *H. armigera* larvae. The six pigeonpea kairomonal components are sesquiterpenes.

In the present study, we demonstrate the important role of the pigeonpea steam distillate components and of a synthetic kairomone composed of a mixture of six pure components for orientational and ovipositional behavior of female *H. armigera*. In addition, we correlate the behavioral responses of the moth to these plant volatiles with an electrophysiological analysis of the perception of the synthetic kairomone and its individual components.

METHODS AND MATERIALS

Insects. A culture of *H. armigera* was maintained in the laboratory at 26–28°C under a light–dark photoperiod of 16:8 hr. The larvae were reared on a

chickpea semiartificial diet as described by Singh and Rembold (1988). Pupae were separated by sex and kept in plastic boxes until eclosion. For mating the 2-day-old adults (six males and five females) were transferred into a mating cage for two days. During the fifth to sixth night after eclosion, those females that had started laying eggs were tested in the wind tunnel.

Test Materials Sampling of the essential oil from the steam distillate of pigeonpea leaves has been described already (Rembold and Tober, 1985). For tests 500 μl of the concentrated pigeonpea steam distillate (500 mg/ml) were diluted 1:500 in *n*-hexane. A synthetic mixture of six biologically active sesquiterpenes, all present in the team distillate, was prepared as a stock solution (Table 1) in *n*-hexane. Rubber septa (7 mm LD, 500-μl volume, Sigma) were used for dispensing the substances. Each septum was filled with 500 μl of test solution and inserted into the test chamber after evaporation of the solvent at room temperature. Septa baited with 500 μl *n*-hexane were used for controls. For tests with pure α-bulnesene and α-humulene, concentrations five times higher than in the synthetic mixture were used. The synthetic kairomone was also tested by controlled release from a glass capillary. Ten microliters of the test solution were pipetted into a glass vial (4 ml, Zinser) equipped with a short microcapillary (1 mm ID, 5 or 15 mm length) in the grid. This glass vial was placed in a three-necked, round-bottomed flask through which air was passed (19 ml/min). This flask was connected by Teflon tube with the test chamber through a stopper mounted in the middle of the upper glass wall (orientation assay). Empty glass vials were used for controls.

TABLE 1. COMPOSITION OF STOCK SOLUTION OF SYNTHETIC PIGEONPEA KAIROMONE MIXTURE IN 1 MILLILITER *n*-HEXANE[a]

Compound	Amount (μl)	Conc. (μg/μl)	PV (%)[b,c]	Significance[c,d]
α-Bulnesene	1	4.9	40.9	***
α-Humulene	4	5.3	27.5	*
α-Guajene	5	1.4	17.5	NS
β-Caryophyllene	6	5.6	8.9	NS
γ-Muurolene	1	8.8	6.5	NS
α-Muurolene	1	6.1	6.5	NS

[a] The six components are listed in order of their decreasing biological activity against *H. armigera* larvae in the olfactometer assay after Rembold et al. (1989).

[b] Preference value (PV) = (larvae with positive − negative olfactometer response/larvae with positive + negative olfactometer response) × 100 (%).

[c] A. Schroth (personal communication).

[d] Statistical significance calculated in the χ^2 test after Sokal and Rohlf (1981) and Precht (1982).

Bioassays. Pigeonpea volatiles were assayed for behavioral activity in a wind tunnel similar to that described by Rembold et al. (1991). The flight tunnel was 60 × 20 × 20 cm, with glass walls on the sides and wire screens at the ends. The room was illuminated with dark red light from two electric bulbs (40 W, M7, Phillips) kept 30 cm above the flight tunnel. The wind velocity was 10 cm/sec, and the temperature was held between 22 and 24°C. All tests were conducted within the first 3 hr after lights off, the time of the main activity of the females.

Orientation Assay. One baited septum was placed in the middle of the test tunnel, 5 cm below the top and covered with black paper. For each test, two 5- to 6-day-old egg-laying females were released into the downwind end of the wind tunnel and observed for 45 min. The following behavioral reactions were counted for each individual female: (1) oriented approaches to the source of odor and antennal contact; (2) tarsal contacts with the baited septum; and (3) circles flown around the odor source.

To quantify the attractiveness of a test substance, an attractivity quotient (AQ) was employed, which was calculated from the total number of positive responses per minute of active flight. The bioassay was statistically evaluated by application of the Wilcoxon, Mann-Whitney ranked test (U test) (Sachs, 1972).

Ovipositional Assay. This was designed as a two-choice test; air (10 cm/sec flow rate) entered the test chamber from both ends and was sucked out in the middle of the test chamber with a fan. Only the sesquiterpene mixture was tested, with *n*-hexane being released at the control end. The baited septa were placed behind the wire screens outside the two ends of the test chamber. In this way direct contact with the test solution was excluded and only olfactory perception and orientation were possible. Two 5- to 6-day-old females were placed in the test chamber. The eggs laid on each wire net were counted after 4 hr and a preference value (PV) was calculated as follows:

$$PV = \frac{\text{eggs on kairomone side} - \text{eggs on control side}}{\text{eggs on kairomone side} + \text{eggs on control side}}$$

The synthetic mixture was applied in three concentrations of 500 μl each, i.e., the stock solution without dilution and as 1:25 and 1:50 dilutions in *n*-hexane. For statistical evaluation, the χ^2-test (Sachs, 1972) was used.

Electrophysiological Methods. Electrophysiological recordings from isolated male and female antennae were performed according to standard methods (Schneider, 1957). In addition to the steam distillate and the six sesquiterpenes, three terpenes (Δ-3-carene, α-pinene, and myrcene) and one alcohol (pentan-1-ol) were tested. The mixture of the latter four compounds is attractive as a chickpea kairomone for *H. armigera* females (Rembold et al., 1991). The two

species-specific sex pheromone components, (Z)-11-hexadecenal (Z11–16:Al) and (Z)-9-hexadecenal (Z9–16:Al), were also tested.

The pure compounds were tested in two dilutions (1:10 and 1:100, in paraffin oil), the steam distillate in seven dilutions ($1:10–1:10^7$, in paraffin oil), and the pheromone compounds in six ($1:10–1:10^6$, in *n*-heptane). Fifty microliters of the solutions were applied on a piece of filter paper (1 × 4 cm), which was inserted into a glass syringe. An airstream of 1-sec duration (airspeed 1 m/sec) was blown over the filter paper. The responses from all the stimuli and doses were standardized to the response of 50 μl of (*E*)-2-hexenal in $1:10^2$ paraffin oil dilution, which was set equal to 1. Each substance was tested with five female and five male antennae.

Calculation of Evaporation Rates. The evaporation rate of all the compounds used for electrophysiological recordings was measured by using headspace adsorption on Tenax GC (Chrompack, Mühlheim) with subsequent gas chromatographic analysis. Fifty microliters of the test substance (each compound diluted $1:10^{21}$ in paraffin oil) was pipetted on filter paper (1 × 4 cm) and placed in a glass syringe (15 cm long, 0.8 cm ID). Air was blown over the filter paper at 50 ml/min for 2 sec, and the evaporated material adsorbed on Tenax GC. The quantity of each component collected on Tenax GC was determined by thermal desorption of the Tenax in a DANI 6200 HR gas chromatograph. This was equipped with a modified flame ionization detector (FID) for splitless injections and was used with a 25-mm × 0.31-mm-ID WCOT fused silica column, helium carrier gas (20 ml/min) and a temperature program of 4°C/min rising from 0°C to 150°C. The method of thermal desorption of compounds from Tenax has been described by Nitz et al. (1984) and Rembold et al. (1989).

RESULTS

Orientation Bioassay. Females exposed to a septum baited with the pigeonpea steam distillate demonstrated two to three times more approaches and tarsal contacts than when offered a control septum (Figure 1). The differences in behavior are statistically significant ($\alpha = 0.01$, Wilcoxon, Mann-Whitney U test; Sachs, 1972). Like the original steam distillate, the synthetic mixture was highly attractive to the females. The frequency of approaches to the synthetic kairomone (1.2 approaches/min) was significantly higher than to the control (0.67 approaches/min) (Figure 2). In contrast to the results from the steam distillate, the number of tarsal contacts showed no clear difference between the synthetic kairomone and the control septum.

Are the two compounds with highest activity or are all six components of the synthetic pigeonpea kairomone mixture (Table 1) equally responsible for its attractivity? The synthetic kairomone was compared with mixtures that were

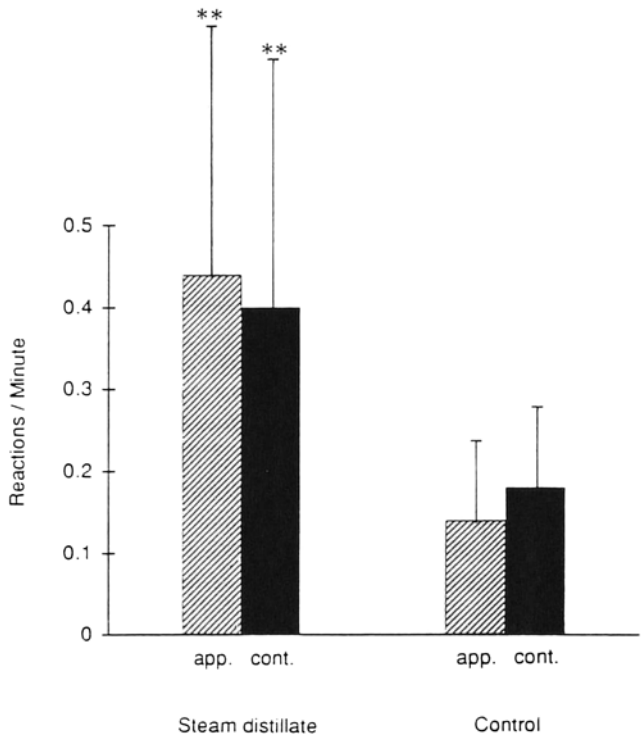

FIG. 1. Behavioral reaction of *H. armigera* females to a pigeonpea steam distillate (N = 10) and to the control (N = 6). Frequency of direct approaches (app.) to the odor source and tarsal contacts (cont.) per minute. **Statistically significant from control at α = 0.01. The bars indicate $\pm$SD.

deficient in the components of highest activity, i.e., α-bulnesene or α-humulene. These two compounds were also tested separately as pure substances. Figure 3 (for α-bulnesene) and Figure 4 (for α-humulene) summarize the results. In the absence of α-bulnesene or α-humulene, the synthetic kairomone lost its activity; the behavioral responses were similar to the control. With pure α-bulnesene the positive responses increased and their number was significantly different from the control (Figure 3). Similar to the synthetic kairomone, the females showed more approaches and antennal contacts (0.95/min) but not more tarsal contacts (0.52/min) to the α-bulnesene-baited septum than to the control (0.67 approaches/

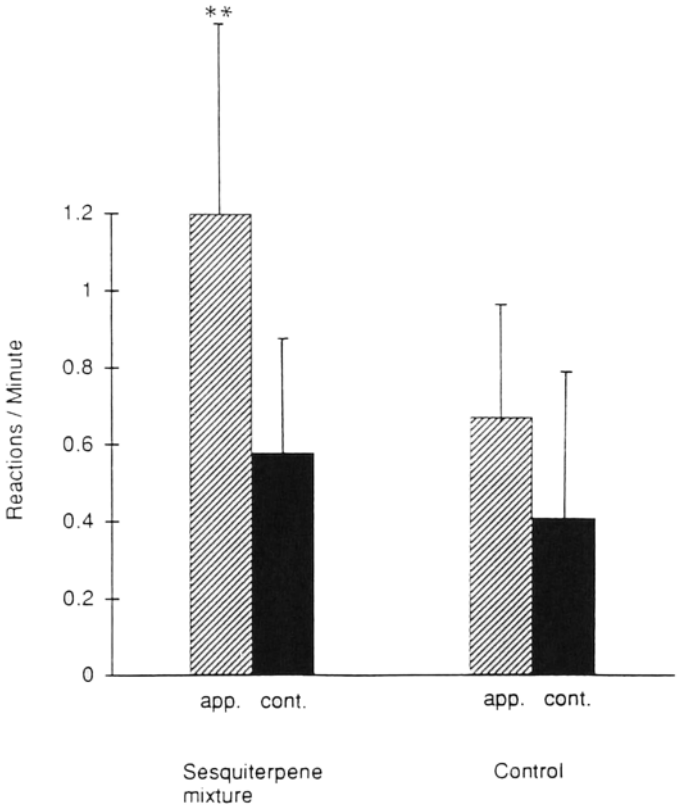

FIG. 2. Behavioral reaction of *H. armigera* females to the sesquiterpene mixture (N = 25) and to the control (N = 38). Frequency of direct approaches (app.) to the odor source and tarsal contacts (cont.) per minute. **Statistically significant from the control at α = 0.01. The bars indicate $\pm$SD.

min, 0.41 tarsal contacts/min). The significance level for the difference in approaches is only $\alpha = 0.05$, which means that α-bulnesene is less active than the whole mixture (significance level $\alpha = 0.01$).

Quite different from α-bulnesene are the responses to the pure α-humulene. There were no additional approaches to this odor source as compared to the control, but the number of tarsal contacts at 0.68/min was significantly higher than to the control at 0.41/min ($\alpha = 0.05$) (Figure 4). Bioassays with a combination of α-bulnesene and α-humulene showed no synergistic effects and no

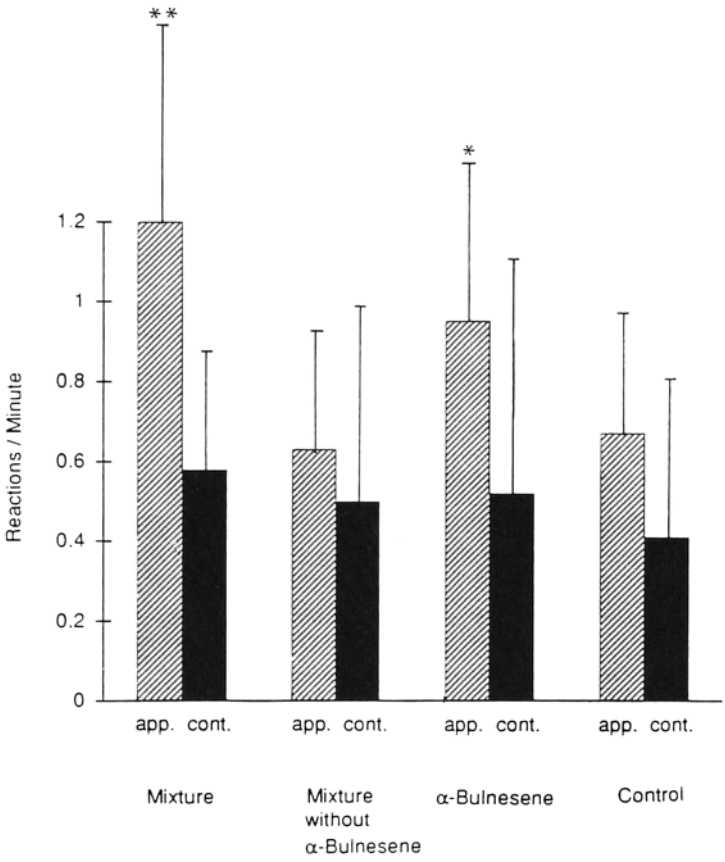

FIG. 3. Frequency of behavioral reactions per minute of *H. armigera* females to the sesquiterpene mixture ($N = 25$), to the mixture without α-bulnesene ($N = 10$), to pure α-bulnesene ($N = 10$), and to the control ($N = 38$); app.: direct approaches to the odor source, cont.: tarsal contacts with the odor source. *Statistically significant from the control at $\alpha = 0.05$; **statistically significant from the control at $\alpha = 0.01$. The bars indicate $\pm$SD.

increase of behavioral responses as compared with the individual substances (Figure 4). A comparison of the attractivity quotients (AQ, total number of positive responses to the test substance per minute flight time) of all the tested odors gives the following sequence of activity. The synthetic kairomone, i.e.,

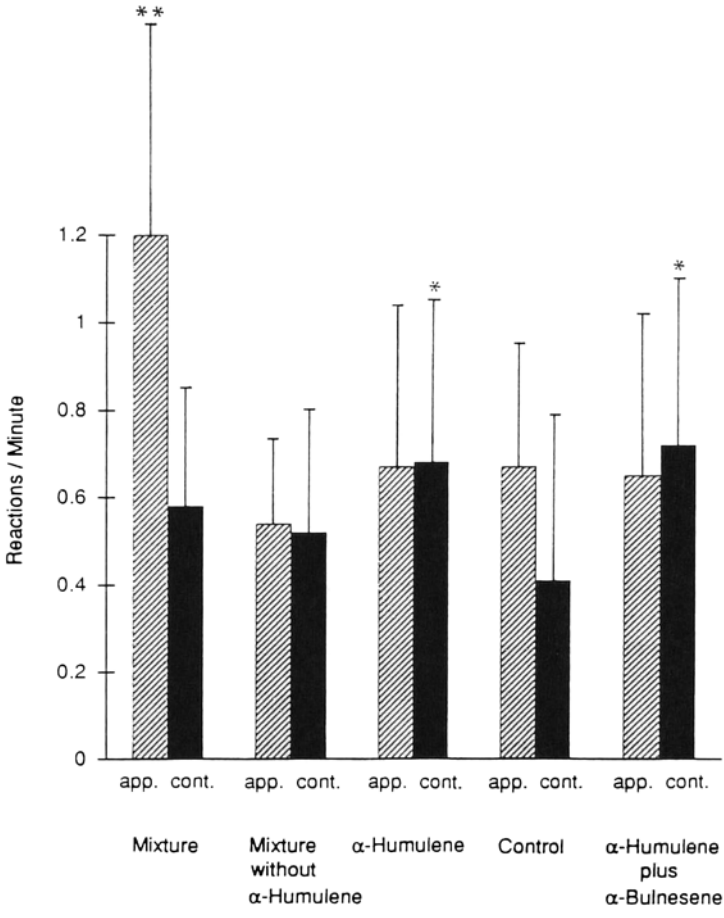

FIG. 4. Behavioral responses of *H. armigera* females to the sesquiterpene mixture (N = 25), to the mixture without α-humulene (N = 12), to pure α-humulene (N = 12), to the control (N = 38), and to the mixture of α-humulene plus α-bulnesene (N = 7). Frequency of direct approaches (app.) to the odor source and tarsal contacts (cont.) with the odor source per minute. *Statistically significant from the control at α = 0.05; **statistically significant from the control at α = 0.01. The bars indicate $\pm$SD.

the whole sesquiterpene mixture (AQ = 1.84) > α-bulnesene (AQ = 1.54) > α-bulnesene plus α-humulene (AQ = 1.5) > α-humulene (AQ = 1.41).

Attraction to the synthetic mixture was concentration dependent. If tested in two relative concentrations (one- and threefold higher) using the capillary method, only the lower concentration was attractive. Females responded to the lower concentration with significantly more approaches per minute (1.24 approaches/min) than to the control (0.47 approaches/min). The number of tarsal contacts was not changed (Figure 5). With the threefold higher concentration

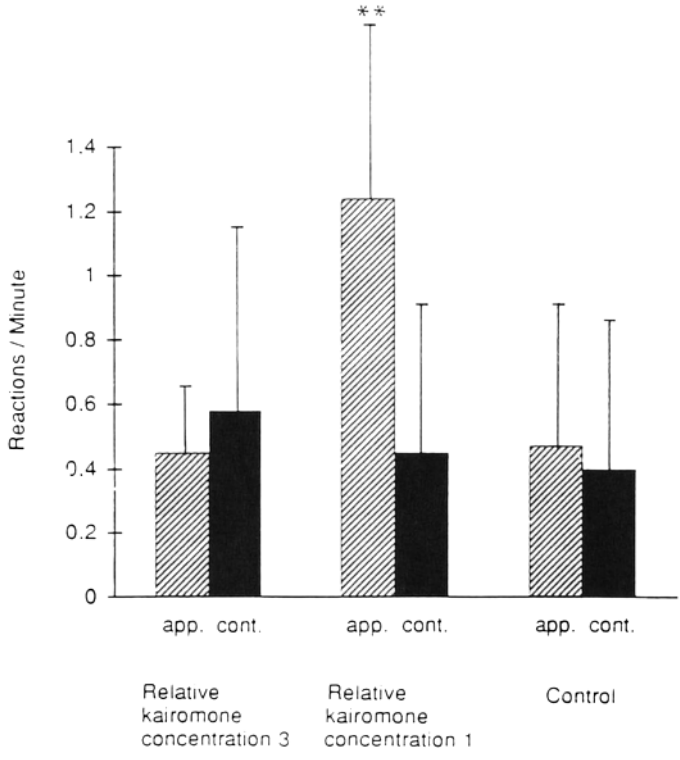

FIG. 5. Behavioral reactions of *H. armigera* females to two different concentrations (relative concentration 1 and relative concentration 3) of the sesquiterpene mixture (N = 8 each) and to the control (N = 16); app.: direct approaches to the odor source, cont.: tarsal contacts with the odor source. **Statistically significant from the control at α = 0.01. The bars indicate ±SD.

TABLE 2. OVIPOSITION TEST WITH THREE DIFFERENT CONCENTRATIONS OF KAIROMONE MIXTURE OF SESQUITERPENES

Kairomone solution	(*N*) Females	Number of eggs			PV (%)[a]	Significance
		Kairomone	Control	Total		
1:50	10	483	263	746	+29.4	0.01
1:25	10	263	315	578	−8.8	NS
1	10	288	435	723	−20.3	0.01

[a]PV = preference value. For calculation see Methods and Materials.

(5 min capillary length) the behavioral responses were not different from the control (Figure 5).

Ovipositional Behavior. The ovipositional assay was designed as a choice test. The influence of the synthetic kairomone on oviposition behavior was concentration dependent (Table 2). Females significantly preferred ($\alpha = 0.01$, χ^2 test) the control side over the stock solution of the sequiterpenes for egg laying [preference value (PV) = −20.3%]. The side with the synthetic sesquiterpene mixture (kairomone) in a 1:50 dilution was preferred (PV = 29.4%), significant at $\alpha = 0.01$. The 1:25 dilution had no effect on the ovipositional behavior since eggs were laid on both sides in nearly the same number (PV = −8.8%).

Electroantennogram. With the plant volatiles, both sexes showed the same electroantennogram responses, but only the male antennae responded to the two individual sex pheromone components (Figure 6A and B). The steam distillate gave a sigmoidal dose–response curve. The distillate elicited much higher electroantennogram responses than its individual compounds or the pheromones at the same concentration. Even at the highest concentration tested (1:10), no saturation in the responses was observed.

Among the individual chemical components, only pentan-1-ol and the three monoterpenes (Δ-3-carene, α-pinene, and myrcene) gave strong EAG responses (Figure 6A and B). The six sesquiterpenes in the 1:10 dilution gave poor activity, and in the 1:100 dilution they produced almost no EAG activity.

The differences between the responses to the individual compounds may have their origin in their different vapor pressures. Quantification of the amount of substance released from the filter paper in 1 sec revealed that the evaporation of pentan-1-ol was twofold higher than that of the three terpenes. The evaporation rate of the terpenes was 20- to 50-fold higher than that of the sesquiterpenes (Figure 7).

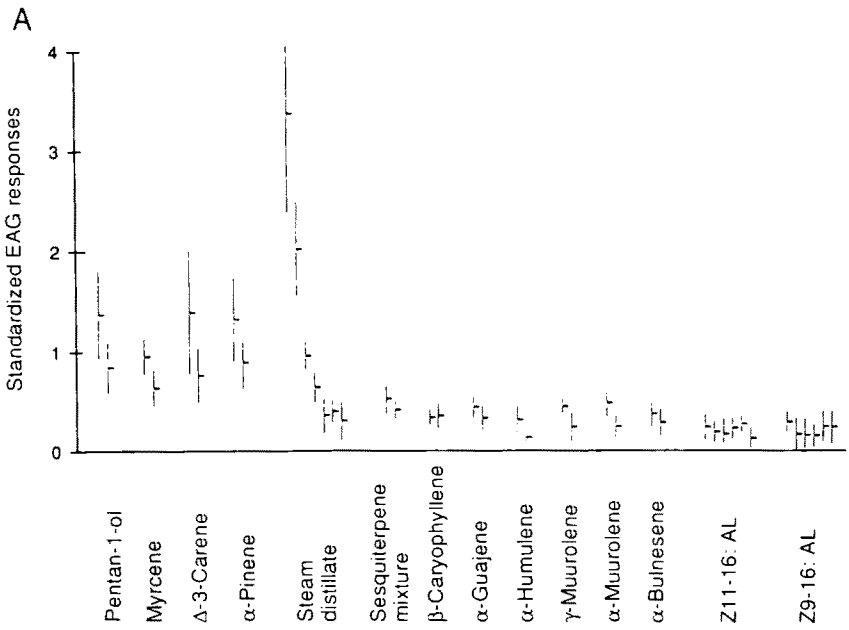

FIG. 6. EAG responses from female (A) and male (B) *H. armigera* antennae, stimulated with different plant volatiles and species-specific pheromone components. Plant volatiles at 1 : 10 and 1 : 100, pigeonpea steam distillate at $1:10–1:10^7$, pheromone components at $1:10–1:10^6$. All responses are standardized based on the response to TRANS-1-hexenal (1 : 100), which is set equal to 1. The bars indicate ±SD.

DISCUSSION

Orientation. Ovipositing *H. armigera* females are strongly attracted by volatiles from a pigeonpea steam distillate, which stimulates two behavioral reactions important for host finding. These are orientation to the odor source from a distance and landing. Several sesquiterpenes were chemically identified in the steam distillate. A mixture of the six sesquiterpenes with the highest attractivity for *H. armigera* first instars (Rembold et al., 1989) was prepared in the same proportion as in the steam distillate and had the same effect on the orientation behavior as the original steam distillate. In contrast to the steam distillate, however, the synthetic mixture of sesquiterpenes did not increase the landing rate of the females compared with the control, indicating that landing might be elicited by several other components. At least some of the six compounds have a synergistic effect, since removal of an individual component (α-

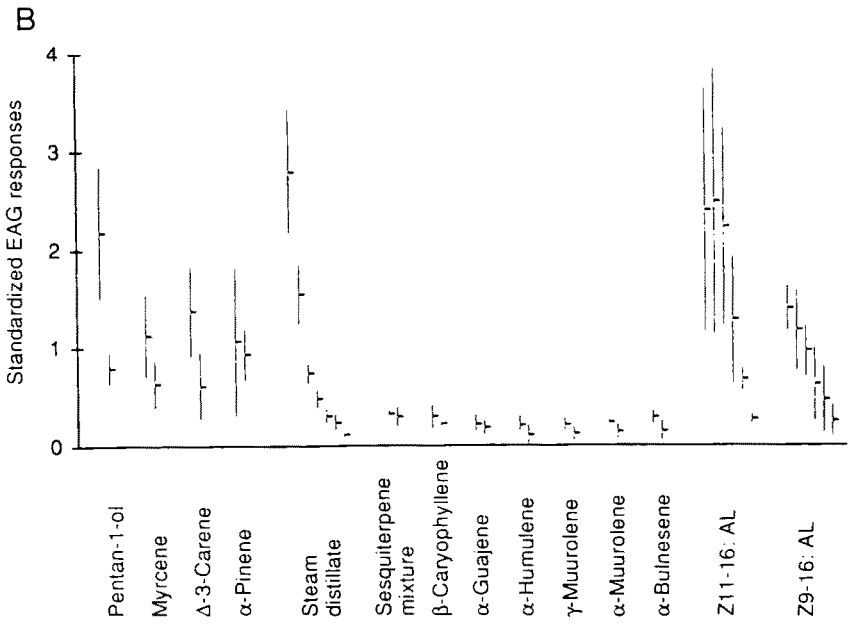

FIG. 6. Continued.

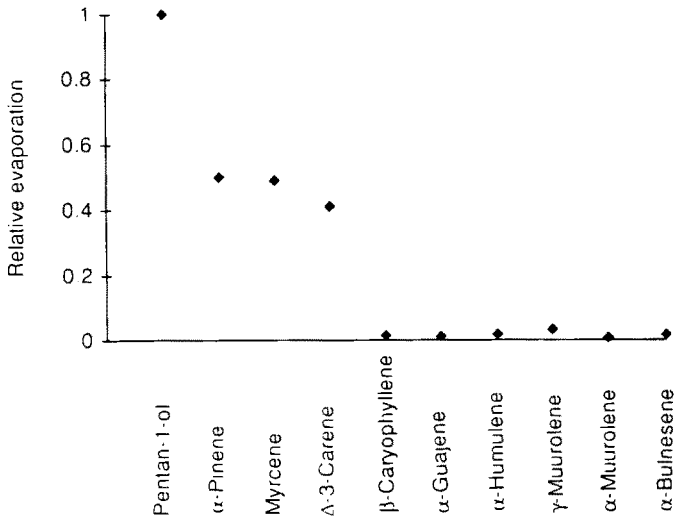

FIG. 7. Relative evaporation of compounds used for the EAG. The release rate of the most volatile compound, pentan-1-ol, is set equal to 1.

bulnesene or α-humulene) resulted in loss of attractivity. α-Bulnesene is the dominant one, as demonstrated by tests with individual compounds in which pure α-bulnesene elicited significantly more approaches than the control, but less than the whole mixture.

Combinations of semiochemicals often are more attractive than individual compounds. Similar results are obtained with the apple maggot fly, *Rhagoletis pomonella* (seven substances; Fein et al., 1982); the carrot fly, *Psila rose* (two substances; Guerin et al., 1983), the beetle *Anthonomus grandis* (five substances; Minyard et al., 1969); and *H. armigera* (four substances; Rembold et al., 1989, 1991). Synergistic effects are not only typical for attractants but have also been found for oviposition-deterring compounds. In *Spodoptera littoralis*, for example, 15 compounds are necessary to elicit a strong deterring effect (Anderson et al., 1993), and in *Ostrinia nubilalis* a mixture of at least five compounds is essential (Thiéry and Le Quere, 1991).

α-Humulene and α-bulnesene stimulate different behavioral responses. α-Bulnesene mainly increased approaches to the odor source, indicating a substance influencing the orientation over long distances, while α-humulene acted at short-distance and positively affected the landing rate. This effect of α-humulene was not visible in the whole mixture and seems to be masked there. With the tse-tse fly, Saini (1980) also found that single compounds and their combinations influenced steps of the searching behavior in different ways.

It is known from preliminary studies that larvae of *H. armigera* are significantly attracted by the sesquiterpene mixture and less strongly by pure α-bulnesene (Rembold et al., in preparation). The same correlation was shown by Rembold et al. (1989, 1991), who found that a mixture of four compounds (three monoterpenes and pentan-1-ol) present in chickpea seed powder was attractive selectively for mated *H. armigera* females (much less for the nonlaying females and not attractive for males) and larvae. Similar olfactory orientation is also known for *Laspeyresia pomonella* larvae and adults in that both stages are attracted to α-farnesene (Sutherland and Hutchins, 1978; Wearing and Hutchins, 1973).

Oviposition. Bioassays demonstrate that the sesquiterpene mixture, in 1 : 50 dilution, stimulates egg laying. The oviposition-stimulating activity of the synthetic mixture is concentration dependent. With increasing concentration, it acts as an oviposition deterrent. Huang et al. (1993) showed, for two *Pieris* species, that their differences in behavioral response to the same substances depended to a large extent on the concentration of the stimuli presented. Similar examples of oviposition stimulants are α-farnesene for the codling moth, *Laspeyresia pomonella* (Wearing and Hutchins, 1973), an ether extract of the host plant for *Papilio demoleus* (Saxena and Goyal, 1978), and dipropyl disulfide for the onion fly, *Delia antiqua* (Harris and Miller, 1988).

Our data suggest that oviposition and orientation are elicited by different optimum stimulus concentrations. The same kairomone concentration that attracts *H. armigera* females is active as an oviposition deterrent, whereas a lower kairomone concentration stimulates egg laying. This selective response reveals that each plant-released stimulus concentration has to be determined experimentally for behavioral response.

Sesquiterpenes have rarely been found as insect attractants or ovipositional stimulants, whereas aliphatic short-chain alcohols and monoterpenes are more common semiochemicals (Smith, 1986; Metcalf, 1988; Renwick and Chew, 1994). Some sesquiterpenes (α-muurolene, γ-muurolene, β-caryophyllene) found in the bark of moribund elm trees (Millar et al., 1986) are attractive to the elm bark beetle, *Scolytus multistriatus*. α-Humulene, α-bulnesene, and β-caryophyllene have been identified in cotton plants (Minyard et al., 1969; Elzen et al., 1985), another host plant of *H. armigera*.

Electroantennograms. Electrophysiological recordings suggest that *H. armigera* females and males respond to plant volatiles in the same way. The steam distillate elicited the strongest EAG responses from antennae of both sexes, while the pheromone components elicited EAG activity only from the male antennae. This finding is consistent with studies with *Agrotis segetum* (Hansson et al., 1989), *Yponomeuta* (Van der Pers, 1981; Van der Pers et al., 1980), and *Pieris rapae* (Topazzini et al., 1990), where females and males responded similarly to plant odor stimuli.

Among the individual compounds, pentan-1-ol and the three monoterpenes gave high EAG responses, and only weak responses were found for the sesquiterpenes. Although the monoterpenes plus pentan-1-ol mixture and the sesquiterpenes mixture are attractive for *H. armigera* females, only the former kairomone mixture gives a clear correlation between EAG responses and behavioral results. There are several examples of semiochemicals that elicit low or no EAG responses (Tichenor and Seigler, 1980; Anderson et al., 1993). A possible explanation may be different component concentrations near the antennae related to the different volatility of alcohols, monoterpenes, and sesquiterpenes. The rate of evaporation of monoterpenes and sesquiterpenes for example, differs by a factor of 20–50. A similar effect was found with single-cell recordings from *Spodoptera littoralis* with aldehydes and terpenes, in which nonanal elicited several times higher EAG responses than the terpenes eugenol and nerolidol in the same doses (Anderson et al., 1993).

An alternative explanation may be that there are fewer receptors for sesquiterpenes on the antenna than for monoterpenes and alcohols. Electrophysiological studies on sex pheromone perception have shown that some components are perceived by a low number of receptor neurons only and therefore give no or very low EAG responses (Löfstedt et al., 1982).

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EFFECT OF PREDATOR ODORS ON HEART RATE AND METABOLIC RATE OF WAPITI (*Cervus elaphus canadensis*)

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Abstract—We measured the heart rate (HR) and oxygen consumption ($\dot{V}_{O_2}$) of wapiti (*Cervus elaphus canadensis*) before, during, and after presentation of biologically irrelevant odors (pentane, thiophene, and a perfume), artificial predator odors (an ether extract of cougar feces, and PDT, a compound found in mustelid anal gland secretion), stale predator odors (dog feces and urine and fox urine, kept at ambient temperature for a few weeks), and fresh predator odors (wolf, coyote, and cougar feces and the odor of a dead coyote, kept frozen between collection and test). Overall, responses to odors were small compared to other stressful stimuli. Individual variability was high among scents and among wapiti, but two of the fresh predator odors (cougar and wolf feces) produced larger HR and $\dot{V}_{O_2}$ responses than the other scents and were more often successful at producing responses. As a group, fresh predator odors produced larger tachycardias and elicited a larger number of significant HR responses than biologically irrelevant novel odors, although the two classes of odors did not differ in their effect on $\dot{V}_{O_2}$. Although several other studies have shown that ungulates have reduced feeding levels when their food is scented with predator odors, it is not clear if this is due to reduced palatability

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or antipredator behavior. This study is the first demonstration that a wild ungulate species reacted more strongly to predator odors than to other odors in a nonfeeding situation.

Key Words—Predator odor, detection, wapiti, elk, heart rate, oxygen consumption, metabolic rate, *Cervis elaphus canadensis*.

INTRODUCTION

The study of predator detection is similar to the study of intraspecific communication in that it requires that the human observer detect a response (or a change in the probability of subsequent behavior) in the receiver of a signal. Responses may include a change in overt activity, such as fleeing, alarm calling, mobbing, or other antipredator behaviors, or can be more subtle, such as a change in foraging level (Sullivan et al., 1985a,b), activity level (Gorman, 1984), habitat selection (Lima and Dill, 1990), or prey survival (Hirsch and Bolles, 1980). Studying the use of chemical cues in predator detection offers the additional challenge, compared with studying visual and acoustic cues, that the signal itself can be difficult to detect by human observers. As a result, there is a paucity of field studies reporting the detection of predators via chemical cues.

Laboratory studies have shown that prey can detect predator odors. Among aquatic organisms, many amphibian larvae and fish avoid waters containing the scent of predatory fish or display antipredator behaviors or a reduced activity level in such waters (Petranka et al., 1987; Martel and Dill, 1993; Stauffer and Semlitsch, 1993), although no nonpredator novel odor was used in these studies. Reactions to predator odors have been demonstrated in the laboratory for many terrestrial species as well. Rats (*Rattus norvegicus*), probably the best-studied mammal, freeze when subjected to the odor of a cat (*Felis domestica*) (Griffith, 1920, cited in Weldon, 1990). Courtney et al. (1968) have shown that this is not a reaction to the novelty of the stimulus: cat odor increased the time it took a trained thirsty rat to run a gangway to a water bottle, while a novel odor (commercial deodorant) had no such effect. Rats also show behavioral reactions typical of fear, as well as increased corticosterone levels (a sign of stress), in response to the odor of fox (*Vulpes vulpes*) feces or of extracts from fox feces (Vernet-Maury, 1980; Vernet-Maury et al., 1984). Geckos (*Coleonyx variegatus*) usually reacted to a 60-sec presentation of a cotton swab rubbed on the sides of a gecko-eating snake with the tail display, which is used almost exclusively during encounters with potential predators, or with flight. Distilled water and a commercial cologne (nonpredator control odor), on the other hand, elicited no tail display or flight. Furthermore, the scent of an arthropod-eating snake resulted in flight on only 30% of presentations. This showed not only that geckos

react to the predator smell, but that they can identify the source of the chemicals as predator (Dial, 1990).

For most other mammalian prey species, however, evidence for predator detection by chemical cues is not as clear. Many laboratory and field studies have shown that herbivores feed less on food items that have been contaminated with predator odors than they do on clean food items (Müller-Schwarze, 1972; Melchiors and Leslie, 1984; Sullivan et al., 1985a,b, 1990a; Sullivan and Crump, 1986; Abbott et al., 1990; Pfister et al., 1990; Robinson, 1990; Merkens et al., 1991; Swihart et al., 1991; Epple et al., 1993).

Although they constitute the ultimate test if an odor is to be used as a repellent, studies of feeding levels are not entirely satisfactory in assessing whether or not responses are due to an association with predators. Dial (1990) stressed the importance of using predator-specific, defensive aspects of behavior as a bioassay to demonstrate that an association with a predator is made. Reduced feeding levels do not demonstrate an association with predators and could be due to neophobia or reduced palatability. In most studies, nonpredator novel odors were also presented, and these reduced feeding for a short while only or not at all, whereas predator odors were effective much longer, especially if they were replenished periodically or if more sophisticated releasing devices were used to slow down evaporation (Sullivan et al., 1985a,b, 1990a; Sullivan and Crump, 1986; Swihart et al., 1991; Epple et al., 1993), thereby ensuring that the responses were not simply due to neophobia. It is more difficult, however, to dismiss the possibility that reduced palatability caused the reduced feeding levels. This possibility is greatest when odors are applied directly on food items, as in Melchiors and Leslie (1984) and in some of the trials of Sullivan et al. (1985b), Abbott et al. (1990), and Swihart et al. (1991). Indeed, reduced palatability could explain why some nonpredator odors did reduce feeding for lengthy periods (e.g., Shumake, 1977; Melchiors and Leslie, 1984; Sullivan et al., 1985b; Conover, 1987; Swihart and Conover, 1990).

Even when odors were presented in various types of containers located close to the food, instead of applied on the food, smell could have reduced the palatability of food (Moulton, 1967; Garcia and Rusiniak, 1980). Indirect evidence for this is provided by the fact that some nonpredator odors were effective in reducing feeding levels even when not released on the food (see, for instance, Abbott et al., 1990; Pfister et al., 1990; Swihart and Conover, 1990). Abbott et al. (1990) found that nonpredator unpleasant odors were less effective than predator odors, but it is difficult to assess what is unpleasant to other species, and some doubts remain regarding an association of predator odors with predators in feeding experiments. This interpretational difficulty can be avoided by not using feeding level as the dependent variable.

Many studies compared trapping success between clean traps and traps contaminated with predator odors (Stoddart, 1980; Dickman and Doncaster,

1984; Gorman, 1984; Robinson, 1990). It should be noted that even in these studies, rodents were attracted to traps with bait, so it cannot be excluded that a reduction of palatability instead of an association with predators caused a reduction in the capture rate of predator-scented traps. Gorman (1984), however, complemented his field study with laboratory experiments that showed, in a non-feeding situation, some rodent species avoided areas with stoat (*Mustela erminea*) scent, and reduced their activity level when surrounded by stoat scent. Other workers showed avoidance by prey species of areas associated with predator odors (Courtney et al., 1968; Pfister et al., 1990; Sullivan et al., 1990b).

To date, only a few species of wild ungulates have been studied for their responsiveness to predator odors and always with the feeding level paradigm. As part of a larger study of the effect of alertness on the relationship between heart rate (HR) and metabolic rate (estimated by rate of oxygen consumption, $\dot{V}_{O_2}$), we had the opportunity to study the effect of predator odors on HR and $\dot{V}_{O_2}$ of wapiti. HR telemetry can be a more sensitive technique than behavioral observation alone to study the reaction of animals to stimuli in their environment, because HR responses are sometimes detected in the absence of overt behavioral responses (Thompson et al., 1968a; Roshchevskii et al., 1976; MacArthur et al., 1979, 1982; Ball and Amlaner, 1980; Ferns et al., 1980; Zimmer, 1982; Geist et al., 1985; Diehl and Helb, 1986). In fact, HR telemetry has already been used to assess the ability of some sounds and objects to repel birds (Thompson et al., 1968a,b; Stout and Schwab, 1980), or of wolf (*Canis lupus*) howls to frighten white-tailed deer (*Odocoileus virginianus*) fawns (Moen et al., 1978). HR has been used a few times to assess whether or not animals reacted to odors, and even to assess which volatiles caused the responses (Wenzel and Sieck, 1972; Hesterman et al., 1976; Goodrich et al., 1978, 1981, 1986, 1990). This technique has already been used to test the effect of predator odors. Rattlesnakes (*Crotalus viridis, C. atrox, C. ruber,* and *C. scutulatus*) increased their HR when exposed to predator odors (Cowles and Phelan, 1958). However, the odor of fox scats did not change the HR of young red deer (*Cervus elaphus*) calves significantly or consistently (Espmark and Langvatn, 1985). It is unclear why the latter study did not reveal a HR response, considering that many acoustic and visual stimuli were effective. Possibly red deer calves are more sensitive to these classes of stimuli than to chemical stimuli. It is clear, however, that HR is ideally suited as a sensitive and easily quantifiable measure of the response of prey animals to predator odors. Moreover, interpretational problems due to possible effects of altered palatability would be avoided if HR changes to predator odors were recorded in a nonfeeding situation.

$\dot{V}_{O_2}$ has very rarely been used to detect responses to stimuli. Floyd (1987) found that visual, acoustic, and tactile stimuli increased both HR and $\dot{V}_{O_2}$ of caribou (*Rangifer tarandus*), and Moritz and Bürgin (1987) found that alarm

pheromones increased $\dot{V}_{CO_2}$ of wasps and bees, which means that $\dot{V}_{O_2}$ also increased. There are reasons to predict that $\dot{V}_{O_2}$ should be less responsive than HR to environmental stimuli: it has been shown that during psychological challenges (such as mental arithmetic, video games, the stress of flying an aircraft, etc.), humans show HR increases that are far larger than what would be expected from the concomitant $\dot{V}_{O_2}$ increases (Blix et al., 1974; Turner and Carroll, 1985; Allen et al., 1986; Sherwood et al., 1986). Yet, stimuli often increased $\dot{V}_{O_2}$ in these studies, and the question of the energetic cost of the reaction of prey to predator odors warrants including $\dot{V}_{O_2}$ in this study.

The objectives of this study were to determine if novel and predator odors evoked HR and $\dot{V}_{O_2}$ responses in captive wapiti in a nonfeeding context, and if predator odors were more effective than novel, biologically irrelevant odors at evoking changes in HR and $\dot{V}_{O_2}$. Furthermore, even though our data are not ideally suited for this task, we investigate if habituation took place.

METHODS AND MATERIALS

Study Site and Study Animals

This study was conducted at the Ministik Wildlife Research Station, 48 km southeast of Edmonton, Alberta, Canada. The station is at the southern fringe of the boreal mixed-wood forest (Renecker, 1987) and occupies an area of about 265 ha, although the wapiti herd is restricted to various pens totaling about 65 ha. The herd contained 25 mature hinds, 13 yearlings and 12 stags in the spring of 1989, when this study was conducted (May 1–June 11). Wapiti grazed on native vegetation but were supplemented with hay and alfalfa–barley pellets, especially during winter. Wild coyotes (*Canis latrans*) are the only predator present in the study area. Six captive female wapiti 6–12 years old, hereafter referred to as Bell, Black, Blue, Ebony, Jasmin, and Teen, were trained to wear a harness-mounted heart-rate transmitter and to stand quietly in a stall while wearing a face mask. The first three were very tame, whereas the remaining animals were habituated to humans.

Measurement of Heart Rate

Heart rate was obtained via radiotelemetry, using an external ECG transmitter stitched to a nylon harness (Johnson et al., 1980; Chabot et al., 1990). Two patterns of lead position allowed ECG recording with good signal-to-noise ratio: both leads on the sternum, about 15 cm apart, or one lead on the sternum and the other on the side of the neck. Instrumentation was routinely performed in 10 min while the animal was standing in a stall. A three-element directional

receiving antenna fed the signal to a receiver (AVM LA-12). The ECG signal was stored on one channel of a stereo tape recorder (Sony TC-158SD or Sony TC-D5) while a verbal description of behavior was stored on the other channel. In the laboratory, the ECG signal was played back into a custom-built decoder/peak detector hooked to a Macintosh Plus microcomputer (Chabot, 1992). Time between R waves (heart period) was measured with millisecond accuracy using a program written in Pascal and timing routines from Rensink (1990). To eliminate errors from the data, beats that lasted less than 65% or more than 17% of the previous beat were flagged. Flagged beats were located, compared to the playback signal, and corrected if necessary. Beats that were unrecoverable were deleted. After correction or removal of erroneous beats, the heart periods were averaged over 1-min intervals, and then transformed into beats per minute (bpm), as prescribed by Chabot et al. (1991).

Measurement of Oxygen Consumption

A continuous-flow indirect calorimetry system, described in detail elsewhere (Chabot, 1992), was used to assess $\dot{V}_{O_2}$ (liters per minute). Fresh outdoor air was allowed into the mask by three large inlets (2.5 cm ID) fitted with plastic hoses about 80 cm long to prevent exhaled air from being lost during forceful expirations. Airflow through the face mask, corrected to standard temperature (0°C) and pressure (101.32 kPa), dry (STPD) (Withers, 1977), was fairly constant (about 480 liters/min) and was measured about every 5 min. Depletion of oxygen in the airstream was measured every 2 sec with a paramagnetic oxygen analyzer (Servomex model 750-b). $\dot{V}_{O_2}$ was calculated using equation 3a of Withers (1977), correcting the flow out of the mask for vapor pressure using wet- and dry-bulb temperatures. Respiratory quotient (*RQ*) was assumed to be 0.87, except for two tests that took place when the subject was fasted for 18 and 37 hr, respectively. *RQ* was assumed to be 0.775 and 0.75 for these two tests (Chabot, 1992). $\dot{V}_{O_2}$ readings were adjusted for the 13-sec lag between a change in oxygen concentration taking place in the mask and its detection by the oxygen analyzer. To eliminate some of the variability due to breathing patterns, the readings of $\dot{V}_{O_2}$ taken every 2 sec were first subjected to a 29-point moving average, and then averaged every minute.

Odors

Feces of cougar (*Felis concolor*) and wolf were collected at the Calgary Zoo on May 15, 1989. Coyote feces were collected in the study area on May 4, 1989. The feces of these three species were collected within 12 hr of being deposited and kept frozen until needed. In addition, a coyote, killed in late April 1989, was kept frozen until it was used in tests on June 3 and 4, 1989. Lastly,

a few "aged" predator odors were collected at a local fox ranch: dog (*Canis familiaris*) urine and feces kept at ambient temperature for many weeks and red fox urine collected overnight on May 28, 1989. These three compounds were kept at ambient temperature until used in tests.

Two artificial predator odors were tested for possible management applications as ungulate repellents. E1 was the ether extract of cougar feces, whereas PDT (3-propyl-1,2-dithiolane), a compound found in anal gland secretions of stoat, wolverine and other mustelids, was synthesized in the laboratory. Both compounds were used undiluted. Three novel scents that are biologically irrelevant, that is, not normally found in the animal's habitat and not related to intra- or interspecific communication, were also tested: pentane, thiophene, and a perfume (Eau de toilette Cléa, 85% vol. 95 N, Yves Rocher 408).

Testing Procedure

Testing of the control and predator odors was performed in a metabolic stall, about 1 m wide, 3 m long, and 2 m high, located outdoors. Subjects already equipped with HR transmitters were fitted with a halter and tied to vertical rails that allowed them to stand or bed, and then equipped with a face mask. A small vacuum pump (Gast 0531-102B-347) brought air from the odor-releasing device to the mask via a small (5-mm-ID) inlet. A two-way brass valve, located between the pump and the odor-releasing device, allowed bypassing the odor-releasing device. All tubing was Teflon to minimize contamination. The distance of about 5 m between the odor-releasing device and the mask resulted in a delay of less than 1 sec between the beginning of a test and the arrival of the scent in the mask.

Two models of odor-releasing devices were used. A two-piece glass cartridge (1 cm ID and 15 cm long) with a Teflon cock at each end (Figure 1) was used with liquid compounds. To prevent contamination, compounds were applied to a piece of filter paper (5 × 30 mm) using a precision pipet and inserted into a 4-cm section of glass tubing. The latter was sealed at both ends with Parafilm until it was inserted into the odor-releasing device, about 1 min before a trial was due to start. The cocks remained closed until 10 sec before testing. To begin a test, the two-way valve was switched from outdoor air to the odor-releasing device for 1 min. At the end of each test the valve was switched back to outdoor air, the stimulus was removed from the cartridge, and the latter flushed with compressed air for about 1 min.

A larger version of the same system (5 cm ID, 40.4 cm long), made of acrylic, was used for fecal samples. Predator feces were mixed with enough distilled water to form a slurry, and the slurry was put directly into the testing device. Whole feces preparations were reused for one to three animals within 3 hr and sometimes used on the next day for a second presentation with the same

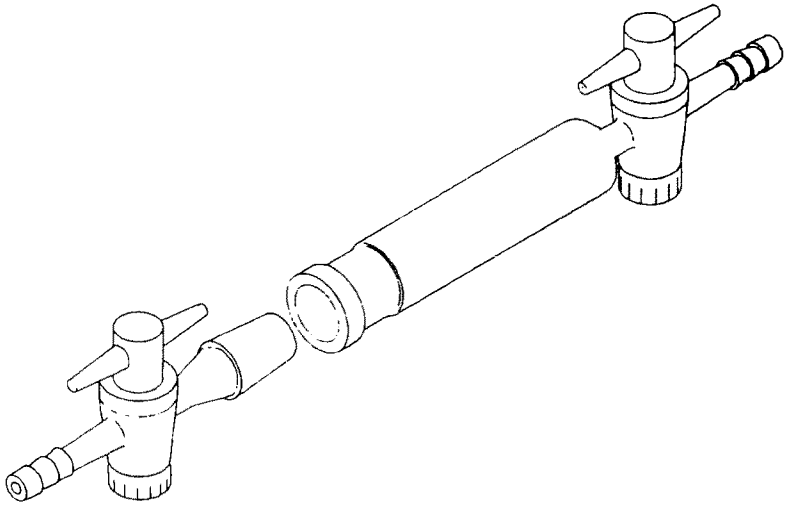

FIG. 1. Odor-releasing device.

animals. The device was washed thoroughly between tests. For the tests involving the dead coyote, the carcass was laid outdoors in the shade, out of view of the subject. No cartridge was used but the pump was connected to a piece of Tygon tubing which picked up air in the animal's fur.

Recording sessions lasted 15–60 min, during which one to six stimuli were applied. Animals were never used in more than two sessions per day. Stimuli were the test odors listed above, a variety of sound playbacks, approaches by a human being, as well as controls with no odor but with the normal toggling of the event recorder's switch at the beginning and end of the 1-min test duration, in case the subjects could hear it. Only the results of control and odor tests are presented here. Attempts were made to obtain 1–2 min of baseline with little or no physical activity before each test, and there was always ≥ 3 min between stimuli. It was impractical to have all test stimuli available at all times for a completely randomized design, but presentation order was random within sessions. There is no information available on wapiti olfaction that would allow the control of perceived stimulus strength across odors; therefore, different concentrations of each odor were attributed quasirandomly across subjects (Table 1). The higher concentrations were easily detected by a human wearing the mask.

Habituation was studied only incidentally: 19 stimulus–animal combinations were tested twice (Tables 1 and 2). On average, time between tests was

TABLE 1. QUANTITY OF EACH TEST ODOR USED FOR FIRST AND, WHEN APPROPRIATE, SECOND PRESENTATION OF EACH COMPOUND TO SIX FEMALE WAPITI

	Pentane (μl)	Perfume (μl)	Thiophene (μl)	E1 (μl)	PDT (μl)	Feces (g)				Urine (μl)		Dead coyote
						Cougar	Coyote	Dog	Wolf	Dog	Fox	
Bell	100		40	10	20	96	"	44/47.7	87	80	40	NQ[b]
Black	40	10	20	10	40/20	96	"	"	175	100	40/40	"
Blue	100	10	60	10/10	10	80/80	33/33	91	"	40	20	NQ
Ebony	100	20	20	20	10/10	96	"	44/47.7	87	60	20	NQ
Jasmin	20	60	20	20	20/10	80/80	33/33	91	175	60/20	40/60	NQ
Teen	100	20	10	10/20	10	80/80	33/33	91	"	40	20	NQ
Mean	76.7	24.0	28.3	13.3/15.0	18.3/13.3	88.0/80.0	33.0/33.0	79.3/47.7	131.0	63.3/20.0	30.0/50.0	NQ

[a] Missing value.
[b] NQ = not quantifiable.

TABLE 2. TIME (HOURS) BETWEEN FIRST AND SECOND PRESENTATION OF ODOR TO SAME ANIMAL

						Feces				Urine			
	Pentane	Perfume	Thiophene	EI	PDT	Cougar	Coyote	Dog	Wolf	Dog	Fox	Dead coyote	Mean
Bell	"	"	"	"	"	"	"	24.7	"	"	"	"	24.7
Black	"	"	"	"	266.8	"	"	"	"	"	11.1	"	139.0
Blue	"	"	"	237.0	"	25.2	20.7	"	"	"	"	21.0	76.0
Ebony	"	"	"	"	215.9	"	"	10.1	"	"	"	"	113.0
Jasmin	"	"	"	"	140.2	22.6	21.7	"	"	173.0	10.8	22.0	65.1
Teen	"	"	"	234.9	"	24.4	19.9	"	"	"	"	19.6	74.7
Mean	"	"	"	236.0	266.8	24.1	20.7	17.4	"	173.0	11.0	21.0	

"Missing value.

80.1 hr (SD = 94.9), with 13 cases tested 10.1–25.2 hr apart, and six cases tested 140.2–266.8 hr apart. Because there was some indication that habituation did occur between trials, only the first presentation of a stimulus to each subject was used to test the effectiveness of the odors.

Statistical Analysis

Assessing Effectiveness of Each Stimulus. HR and $\dot{V}_{O_2}$ were measured before (min −1), during (min 0), and after (min 1) stimulus presentation. Small sample sizes ($N \leq 6$) made it difficult to test the assumptions of univariate or multivariate repeated-measures ANOVA; therefore we used two paired-sample t tests (Zar, 1984) for each dependent variable: one to test for an increase in HR or $\dot{V}_{O_2}$ between min −1 and min 0, and another to test for an increase between min −1 and min 1. Because increases were predicted, one-tailed tests were used. These two comparisons were nonorthogonal, so a level $\alpha = 0.05$ of type I error was maintained by requiring each comparison to be significant at $1 - (1 - \alpha)^{0.5} = 0.025$ (Šidák's multiplicative inequality; Kirk, 1982, p. 110). In the Results section, figures show means with 95% confidence intervals. The one-tailed t test described here is closely approximated by looking for 95% confidence intervals that do not include 0.

A second method of measuring the effectiveness of the odors at increasing HR or $\dot{V}_{O_2}$ was also devised. We computed two new variables, ΔHR and $\Delta\dot{V}_{O_2}$, which are the difference scores between consecutive minutes of recording (MacArthur et al., 1979). The distribution of these two variables for calm animals was assessed using data for the same six animals standing or bedded, and at least 3 min after a change of activity or presentation of a stimulus. For ΔHR, pairwise Kolmogorov-Smirnov tests showed that the distributions were not statistically different for Bell, Black, and Blue, and for Ebony, Jasmin, and Teen. Thus ΔHRs were pooled in two groups. Neither group was normally distributed (Lillifors' test, $P < 0.001$), and both were leptokurtotic (Table 3). Therefore, critically high values ($P = 0.05$, one-tailed) were calculated empirically by ranking ΔHRs for each group in decreasing order and taking the fifth percentile ΔHR. For Bell, Black, and Blue, HR increases of 2.4 bpm or more between subsequent minutes of recording occurred less than 5% of the time when they were calm. For Ebony, Jasmin, and Teen, this number is 3.77 bpm. The same procedure was followed for $\Delta\dot{V}_{O_2}$, except that the distribution of this variable across all six animals was similar enough to pool the data. The distribution of $\Delta\dot{V}_{O_2}$ was normal (Table 3), and increases in $\dot{V}_{O_2}$ of 0.2857 liter/min or more occurred less than 5% of the time.

The effectiveness of a stimulus at increasing HR or $\dot{V}_{O_2}$ was assessed by taking the number of effective and ineffective stimulus presentations, according to the criteria of Table 3, and converting to a probability with the binomial test ($P = 0.05$, $q = 0.95$). This technique, only available for min 0 (i.e., during

TABLE 3. DECRIPTIVE STATISTICS, TEST OF NORMALITY, AND EMPIRICAL CRITICAL VALUES FOR ΔHR AND $\Delta \dot{V}_{O_2}$

Variable	Animals	N	Min	Max	Mean	SD	g_1	g_2	Lilliefors test of normality		Critical value 0.05, one-tailed
									Maxdif	P	
ΔHR	Bell, Black, Blue	695	−6.082	5.875	−0.178	1.644	−0.084	1.437	0.068	0.000	2.400
ΔHR	Ebony, Teen, Jasmin	640	−9.762	11.799	−0.263	2.472	0.108	2.283	0.062	0.000	3.768
$\Delta \dot{V}_{O_2}$	all six	1560	−0.559	0.712	0.001	0.172	0.084	0.335	0.021	0.122	0.286

stimulus), is less affected by individual differences in responsiveness and by HR or $\dot{V}_{O_2}$ variability than the paired-sample t test, and took advantage of large samples for the distribution of these two variables in calm animals. The t test, however, is more appropriate to detect small but consistent changes in HR or $\dot{V}_{O_2}$ due to the test stimuli and is the only method to detect significant changes in HR or $\dot{V}_{O_2}$ in the minute following stimulus presentation.

Comparing Odor Groups.

There were too many different stimuli relative to the number of subjects for a statistical comparison of the effectiveness of stimuli among each other. Instead we combined the odors into four classes: biologically irrelevant (pentane, perfume, thiophene), artificial (El and PDT), stale predator odors (fox urine, dog urine and feces), and fresh predator odors (cougar, coyote, and wolf feces; dead coyote). In a first test, each odor presentation was scored 1 if it was effective at increasing HR, and 0 otherwise. The scores of the odor groups were then compared using Fisher's exact tests. The same analysis was repeated with $\dot{V}_{O_2}$. A repeated-measures ANOVA was also performed on ΔHR and $\Delta\dot{V}_{O_2}$ for each animal, averaged across the odors of each class. Odor class (four levels) was the within-subject factor. Using the criteria of Potvin et al. (1990), we selected the univariate test corrected with Huynh-Feldt's ϵ.

Effect of Environmental and Physiological Variables. Pooling data from all subjects and odors, stepwise multiple regression (Pedhazur, 1982) with α-to-enter and α-to-remove set to 0.015 (Wilkinson, 1989) was used to test if the size of responses (ΔHR or $\Delta\dot{V}_{O_2}$) was affected by date, time of day, baseline HR, order of presentation within trial, an index of agitation, weight, number of days pregnant, number of days lactating, and ambient temperature.

Stimulus Intensity. For each odor, the effect of stimulus intensity on HR or $\dot{V}_{O_2}$ responses was assessed by calculating Spearman rank correlation coefficients between quantity used and ΔHR and $\Delta\dot{V}_{O_2}$ (Zar, 1984).

Habituation. To test for habituation, ΔHR at min 0 for the first and second presentation of an odor to an animal were compared with paired-sample t tests (one-tailed), combining all odors and animals. The same method was used with $\Delta\dot{V}_{O_2}$. In addition, the number of effective trials upon first and second presentation was compared using Fisher's exact test (one-tailed).

Contingency tables were calculated in SAS (SAS Institute, 1989), t tests in SuperAnova (Abacus Concepts, 1989), and the remaining statistical tests were done with Systat 5.0 (Wilkinson, 1989).

RESULTS

Only cougar feces, wolf feces, and dog urine increased HR significantly during stimulus presentation [paired-sample t test, P (one-tailed) < 0.025; Table

TABLE 4. EFFECT OF TEST ODORS ON HEART RATE (HR) OF SIX WAPITI HINDS

		Mean HR			min −1 vs min 0					min −1 vs min 1		
Stimulus	*N*	min −1	min 0	min 1	Mean ΔHR	SE	Paired *t* test (one-tail)	*N* above critical value	Binomial test	Mean ΔHR	SE	Paired *t* test (one-tailed)
Control	6	50.77	50.53	50.08	−0.24	0.517	>0.05	0	0.735	−0.69	1.510	>0.05
Pentane	6	63.99	64.62	64.28	0.63	2.171	>0.05	2	**0.031**	0.29	1.463	>0.05
Perfume	5	52.83	53.64	53.32	0.81	0.424	>0.05	0	0.734	0.50	0.936	>0.05
Thiophene	6	61.10	61.93	61.31	0.83	1.113	>0.05	1	0.232	0.21	1.080	>0.05
E1	6	60.80	60.25	60.56	−0.56	0.832	>0.05	0	0.735	−0.25	1.059	>0.05
PDT	6	56.80	59.81	57.68	3.01	1.302	0.035	2	**0.031**	0.88	1.318	>0.05
Fox urine	6	59.82	60.79	59.97	0.98	0.386	0.026	0	0.735	0.16	0.850	>0.05
Dog urine	6	57.80	60.39	56.84	2.59	0.466	**0.002**[a]	2	**0.031**	0.17	0.744	>0.05
Dog feces	5	53.28	56.90	54.66	3.61	2.078	>0.05	2	**0.021**	1.38	1.562	>0.05
Cougar feces	6	55.71	61.03	58.08	5.32	1.400	**0.007**	5	**0.000**	2.36	1.290	>0.05
Coyote feces	3	55.52	56.29	57.62	0.77	3.446	>0.05	1	0.135	2.11	3.888	>0.05
Wolf feces	4	59.05	65.21	64.54	6.16	1.745	**0.020**	3	**0.001**	5.49	1.767	0.027
Dead coyote	5	56.93	57.73	56.72	0.81	1.183	>0.05	0	0.774	−0.21	0.322	>0.05

[a]Values in boldface type indicate significant increases in HR.

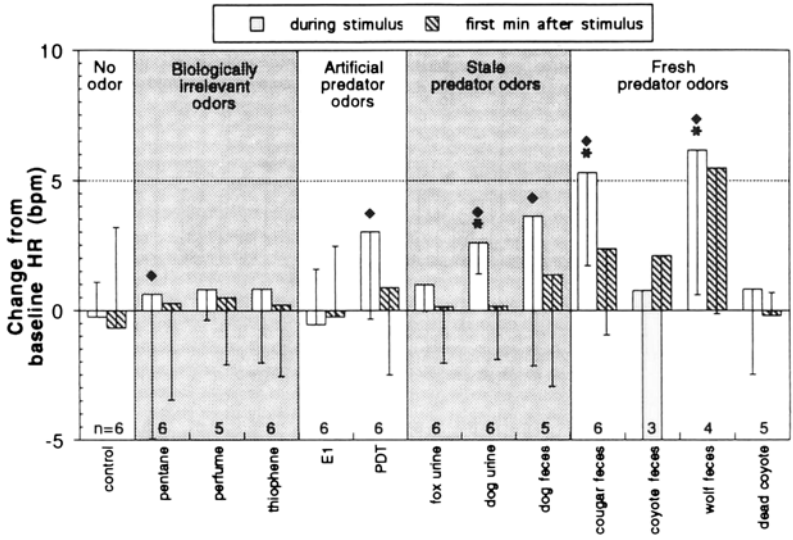

FIG. 2. Change in heart rate, with 95% confidence interval, produced by the test stimuli. Odors found significant with paired-sample t tests are marked with an asterisk, while those found significant with the binomial tests are marked with a diamond. Sample size for each odor is indicated at the bottom of the figure.

4 and Figure 2), although tachycardia was nearly significant for PDT and fox urine ($0.026 \leq P \leq 0.035$). HR during the first minute after stimulus did not differ from baseline HR, although tachycardia was nearly significant with wolf feces ($P = 0.027$; Table 4). All odors found significant with a paired-sample t test were also significant with the binomial test (Table 4), but three more odors, pentane, PDT, and dog feces, were effective when analyzed with the binomial test (Table 4).

$\dot{V}_{O_2}$ during stimulus presentations did not differ from baseline according to paired-sample t tests, but with binomial tests, six odors were found effective at increasing $\dot{V}_{O_2}$: pentane, cougar, dog and wolf feces, dog urine, and the dead coyote (Figure 3, Table 5). $\dot{V}_{O_2}$ during the first minute after exposure to cougar and wolf feces was significantly elevated relative to the prestimulus level (Figure 3, Table 5).

HR responses were not affected by the environmental variables measured (date, time of day, baseline HR, order of presentation within trial, index of agitation, weight, number of days pregnant, number of days lactating, ambient temperature), as the stepwise regression resulted in no variable being entered.

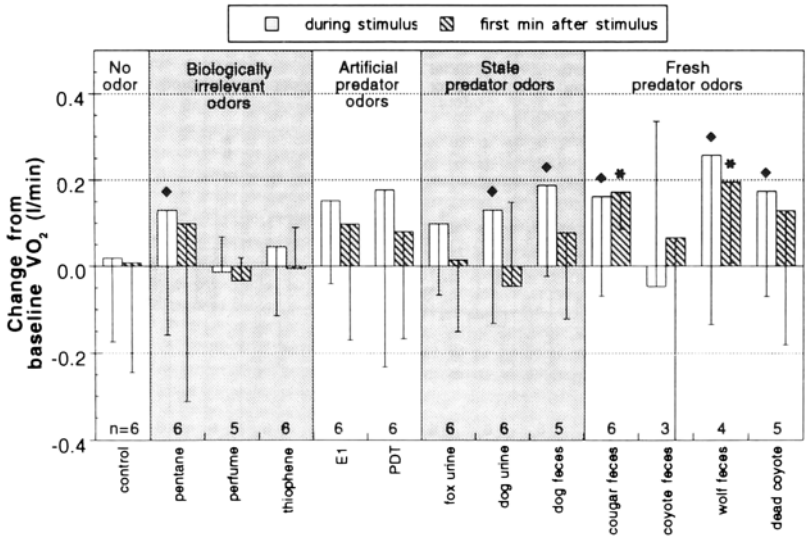

FIG. 3. Change in oxygen consumption, with 95% confidence interval, produced by the test stimuli. Odors found significant with paired-sample *t* tests are marked with an asterisk, while those found significant with the binomial tests are marked with a diamond. Sample size for each odor is indicated at the bottom of the figure.

However, agitation during stimulus presentation resulted in larger $\Delta\dot{V}_{O_2}$:

$$\Delta\dot{V}_{O_2} = 0.066 + 0.024 \text{ Agit } (R^2 = 0.111, P = 0.005)$$

where Agit is an index of agitation ranging from 0 to 15 (mean = 1.8, SD = 2.8).

With the quantities used, there was no evidence that odor strength affected the size of the response. Spearman rank correlation coefficients between quantity used and either ΔHR or $\Delta\dot{V}_{O_2}$ were not significant, although these negative results are likely due to the very small sample sizes.

There was some evidence for habituation. For those animal–stimulus combinations that were tested twice, HR and $\dot{V}_{O_2}$ responses were smaller on the second testing than the first, both during the stimulus presentation and during the following minute. However, this decline was significant only for HR [paired-sample *t*-test (one-tailed), $P = 0.014$ (min 0) and 0.042 (min 1); Table 6]. There was also a reduction in the number of presentations resulting in significant tachycardias (6 of 19 trials vs. 3 of 19 trials) or significant $\dot{V}_{O_2}$ increases (5 of 19 trials vs. 1 of 19 trials; Table 6), but this was not significant [Fisher's exact

TABLE 5. EFFECT OF TEST ODORS ON OXYGEN CONSUMPTION ($\dot{V}_{O_2}$) OF SIX WAPITI HINDS

					min-1 vs min 0					min 1 vs min 1		
		Mean $\dot{V}_{O_2}$			Mean		Paired	*N* above		Mean		Paired
Stimulus	*N*	min −1	min 0	min 1	$\Delta\dot{V}_{O_2}$	SE	*t* test (one-tailed)	critical value	Binomial test	$\Delta\dot{V}_{O_2}$	SE	test (one-tailed)
Control	6	1.46	1.48	1.47	0.019	0.075	>0.05	0	0.735	0.008	0.099	>0.05
Pentane	6	2.01	2.14	2.11	0.130	0.112	>0.05	3	**0.002**	0.009	0.160	>0.05
Perfume	5	1.54	1.53	1.51	−0.013	0.029	>0.05	0	0.774	−0.034	0.020	>0.05
Thiophene	6	1.96	2.00	1.95	0.045	0.062	>0.05	1	0.232	−0.005	0.037	>0.05
E1	6	1.84	1.99	1.93	0.152	0.075	0.049	1	0.232	0.098	0.104	>0.05
PDT	6	1.87	2.04	1.95	0.177	0.159	>0.05	1	0.232	0.080	0.096	>0.05
Fox urine	6	1.81	1.91	1.83	0.098	0.064	>0.05	1	0.232	0.014	0.064	>0.05
Dog urine	6	1.96	2.09	1.91	0.130	0.102	>0.05	2	**0.031**	−0.047	0.070	>0.05
Dog feces	5	1.76	1.95	1.84	0.187	0.076	>0.036	2	**0.021**	0.077	0.071	>0.05
Cougar feces	6	1.91	2.07	2.08	0.162	0.090	>0.05	3	**0.002**	0.172	0.034	**0.002**
Coyote feces	3	1.55	1.50	1.61	−0.047	0.089	>0.05	0	0.857	0.066	0.447	>0.05
Wolf feces	4	1.87	2.13	2.07	0.258	0.123	>0.05	2	**0.014**	0.195	0.059	**0.023**
Dead coyote	5	1.70	1.87	1.83	0.174	0.088	>0.05	2	**0.021**	0.129	0.112	>0.05

[a] Values in boldface type indicate significant increases in $\dot{V}_O$

TABLE 6. DIFFERENCE IN NUMBER OF EFFECTIVE RESPONSES AND SIZE OF HR AND $\dot{V}_{O_2}$ RESPONSES BETWEEN FIRST AND SECOND PRESENTATION OF STIMULUS TO SAME ANIMAL[a]

	First presentation			Second presentation				
	N effective	Mean change	SE	*N* effective	Mean change	SE	*t*	*P*
HR, min 0 vs min −1	6	2.64	0.953	3	0.42	0.433	2.392	0.014
HR, min 1 vs min −1		1.38	0.800		−0.29	0.367	1.830	0.042
$\dot{V}_{O_2}$, min 0 vs min −1	5	0.15	0.060	1	0.07	0.043	1.325	0.101
$\dot{V}_{O_2}$, min 1 vs min −1		0.06	0.044		0.04	0.045	0.415	0.341

[a]Odors were deemed effective when they produced changes in HR or $\dot{V}_{O_2}$ meeting the criteria of Table 3. Mean change in HR or $\dot{V}_{O_2}$ were based on all stimuli ($N = 19$). Means were compared with a paired-sample *t* test (one-tailed).

test, $P = 0.22$ (HR) and 0.09 ($\dot{V}_{O_2}$)]. There were three cases of reversal: for Blue with E1, tachycardia was significant on the second but not the first presentation, whereas for Jasmin with dog urine and Black with PDT, a significant $\Delta\dot{V}_{O_2}$ was found on the second but not the first presentation. Interestingly these three cases all had ≥ 140 hr between tests.

Our data show large individual variability in response rate. Figure 4 compares the frequency of significant tachycardia (using the criteria of Table 3) each animal produced in response to the four classes of odors (the no-odor controls were excluded from this analysis). One animal (Blue) never responded, while another (Bell) responded to at least half of the stimulus presentations in all four stimulus classes. The other animals fell in between these two extremes. Overall (i.e., pooling all odor classes), the six hinds differed significantly in their response rate ($\chi^2 = 11.3$, $P = 0.046$). Individual variability was likewise high with $\dot{V}_{O_2}$, although the difference was not significant ($\chi^2 = 7.5$, $P = 0.189$). Again Blue did not respond to any stimulus (Figure 5). No animal had significant responses in all four odor categories, and some hinds responded to stimuli that did not affect other animals.

Artificial odors affected the fewest animals: two hinds for HR (Figure 4), and a single subject for $\dot{V}_{O_2}$ (Figure 5). Biologically irrelevant odors affected three animals on both dependent variables. Three hinds had HR responses to stale predator odors, whereas four hinds showed $\dot{V}_{O_2}$ responses to these odors. Fresh predator odors had a more general effect, producing HR and $\dot{V}_{O_2}$ reactions in all animals but Blue, even if individual response rates were not higher than for other categories for which an animal also responded. On average, the six hinds responded to 50% of the fresh predator odors with significant tachycardia,

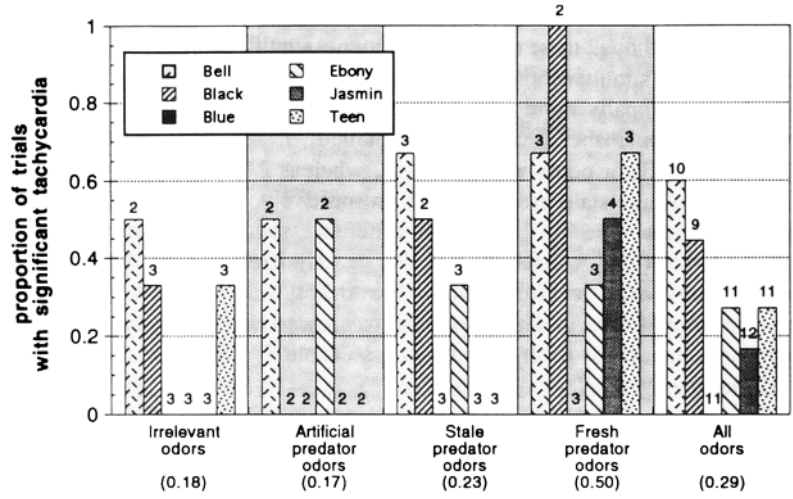

FIG. 4. Rate HR responses of each animal for the four odor categories and for all odors combined. Sample sizes are given above each bar, and the mean percentage of response is given at the bottom.

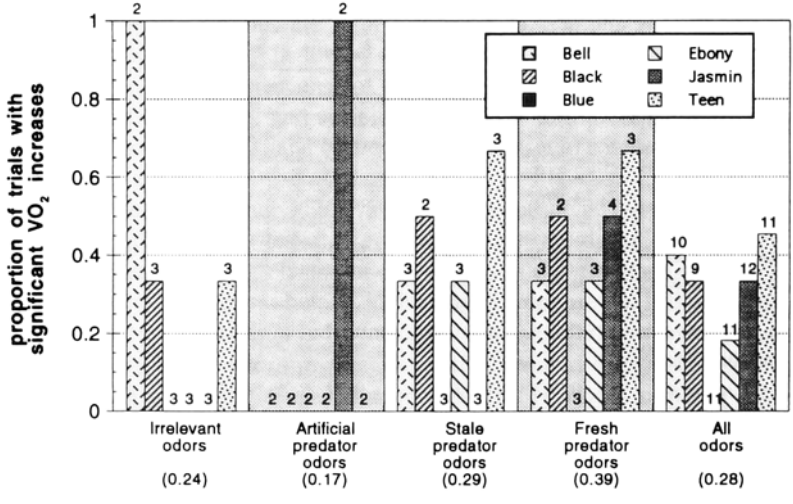

FIG. 5. Rate of V_{O_2} responses of each animal for the four odor categories and for all odors combined. Sample sizes are given above each bar. Sample sizes are given above each bar, and the mean percentage of response is given at the bottom.

but only to 18, 17, and 24% of the irrelevant, artificial, and stale predator odors, respectively. Although these differences were not significant when all four classes were compared simultaneously ($G^2_{[3]} = 5.89$, $P = 0.18$), fresh predator odors elicited proportionally more effective tachycardias than the biologically irrelevant novel odors [Fisher's exact test (one-tailed), $P = 0.047$]. With $\dot{V}_{O_2}$, 39% of the fresh predator odors were effective, whereas 24, 17, and 28% of irrelevant, artificial, and stale predator odors, respectively, had an effect. However, these differences were not statistically different, whether all four odor classes were compared ($G^2_{[3]} = 2.03$, $P = 0.57$), or only irrelevant and fresh predator odors [Fisher's exact test (one-tailed), $P = 0.27$].

The average size of HR responses was lowest for biologically irrelevant smells and highest for fresh predator odors (Table 7). We were unable to discriminate among the four odor classes ($F_{(3,15)} = 2.24$, $P_{\text{H-F adjusted}} = 0.13$). However, fresh predator odors produced larger tachycardias than biologically irrelevant novel odors ($t = 2.26$, $P_{\text{H-F adjusted}} = 0.037$). The size of $\dot{V}_{O_2}$ responses did not differ between odor classes, whether all classes were compared or just

TABLE 7. AVERAGE SIZE OF HEART RATE AND $\dot{V}_{O_2}$ RESPONSES IN FOUR ODOR CLASSES[a]

	Change from baseline		Comparison among four odor classes		Irrelevant odors vs fresh predator odors	
Odor class	Mean	SE	$F_{(3,15)}$	P (H-F adjusted)	$t_{(5)}$	P (one-tailed)
Heart rate, minute 0 (bpm)						
Biologically irrelevant	0.904	0.735	2.24	0.13	2.26	0.037
Artificial predator odors	1.226	0.978				
State predator odors	2.319	0.783				
Fresh predator odors	3.676	1.089				
$\dot{V}_{O_2}$, minute 0 (liter/min)						
Biologically irrelevant	0.077	0.068	0.27	0.76	0.75	0.24
Artificial predator odors	0.164	0.110				
Stale predator odors	0.134	0.047				
Fresh predator odors	0.143	0.038				
$\dot{V}_{O_2}$, minute 1 (liter/min)						
Biologically irrelevant	0.042	0.076	0.99	0.41	1.65	0.08
Artificial predator odors	0.089	0.096				
Stale predator odors	0.009	0.040				
Fresh predator odors	0.140	0.033				

[a] $\dot{V}_{O_2}$ responses for the minute following scent presentations are also compared because $\dot{V}_{O_2}$ was sometimes significantly elevated then. All responses for each subject were averaged within odor classes, then these averages were themselves averaged across animals.

irrelevant odors and fresh predator odors. The same was true for $\dot{V}_{O_2}$ in the minute following stimulus (Table 7).

DISCUSSION AND CONCLUSIONS

HR and $\dot{V}_{O_2}$ Responses to Odors

Averaging both HR and $\dot{V}_{O_2}$ over 1 min reduced the sensitivity of the analysis. Sometimes a smell induced a few rapid heartbeats near the beginning of a test, but yielded a nonsignificant ΔHR. Such transient HR changes, while indicating that the subject has detected the stimulus, are likely unimportant energetically. Changes in HR or $\dot{V}_{O_2}$ large enough to affect 1-min averages constitute better evidence of increased alertness beyond detection of the stimulus, such as one would expect if associations were made with predators. Other advantages of using 1-min averages were the elimination of much natural variability in the data, and a more even comparison between both dependent variables, which follow different time courses.

The kind of individual variability observed here is not unusual (see for instance, Wenzel and Sieck, 1972). Blue did not respond to any chemical stimulus (although she did respond to some of the other stimuli; Chabot, 1992). She was the dominant female in the herd, and she was an extremely tame and calm animal. Black, and to a lesser degree Bell, were also very calm animals, so calmness alone does not explain why Blue did not respond to any stimulus. Other possible explanations are prior history of the various animals, because exposure to an odor early in life in association with a pleasant or unpleasant experience can affect the response of test animals to the same compound alone in later life (Doty, 1986), and social status, which can affect both HR (Cherkovich and Tatoyan, 1973) and corticosterone levels and could also affect reactivity to mild stimuli.

One effect of the individual variability we observed was to reduce the sensitivity of the paired *t* tests: a few responses in the opposite direction can prevent the detection of a change in HR or $\dot{V}_{O_2}$ in the group of subjects. The alternative binomial test was helpful in this context because it relied on assessing the effectiveness of each trial independently, with no interference from ineffective trials. Overall, 70 odor presentations with six wapiti elicited 18 significant ΔHRs, and 18 significant $\Delta\dot{V}_{O_2}$. Moreover, in 10 cases both variables showed a significant change simultaneously. Two of the odors, cougar and wolf feces, had a stronger effect than the others: mean HR changes were significant and were highest among the 12 odors tested. Cougar and wolf feces were also the only two smells that produced a significant change in mean $\dot{V}_{O_2}$, although this was recorded in the first minute after stimulus instead of during the stimulus. These same two scents also produced the highest proportion of effective trials

(Table 3): with cougar feces, five of six animals showed significant tachycardia, while three of six showed significant increases in $\dot{V}_{O_2}$. Wolf feces affected three of four animals for HR, and two of four animals for $\dot{V}_{O_2}$. Finally, these were the only two odors that were found effective for both dependent variables with both statistical tests (paired-samples *t* test and binomial test). That wapiti responded to wolf and cougar feces with increases in both HR and $\dot{V}_{O_2}$ in a nonforaging context is the first clear demonstration that wild ungulates react to predator odors for reasons other than reduced food palatability.

The other odors appeared less effective than wolf and cougar feces. Two stale predator odors, dog feces and urine, elicited a significant number of HR and $\dot{V}_{O_2}$ responses but affected fewer animals than wolf and cougar feces, and dog urine was found to increase HR significantly by the *t* test (Figure 2). Pentane produced significant increases in both HR and $\dot{V}_{O_2}$ for Bell and Black, the only biologically irrelevant odor to affect both variables in the same subjects. It also increased Teen's $\dot{V}_{O_2}$. Two other smells were effective but with only one of the two dependent variables: the smell of a dead coyote increased $\dot{V}_{O_2}$ in two of six animals, which was significant, whereas PDT, found in anal gland secretions of many mustelids, elicited significant tachycardia in two animals (Bell and Ebony), and increased $\dot{V}_{O_2}$ in another animal (Jasmin). This single $\dot{V}_{O_2}$ response did not lead to a significant binomial test.

Some of these differences could be due to differences in stimulus intensity, which we were unable to equate for the various odors because of the lack of knowledge of wapiti chemoreception. Furthermore, odors in concentrations that differ markedly from those encountered by the animals under natural conditions could release false physiological as well as behavioral reactions (Goodrich et al., 1981) Predator odors were usually presented in large quantities, but irrelevant and artificial predator odors were highly concentrated. The best we could do was to ensure that all odors were detectable in the mask by the observer, at least at the highest intensity for each odor.

Although a statistical comparison of the effectiveness of each odor was impossible due to the large number of odors relative to the number of subjects, we were able to show that the four general classes of odors (biologically irrelevant odors, artificial predator odors, stale predator odors, and fresh predator odors) did not differ in either the size of HR and $\dot{V}_{O_2}$ responses they produced or in the proportion of effective trials they elicited. Possibly, a larger number of subjects would have allowed discrimination among these groups, at least for HR. However, two of these odor groups, the artificial predator odors and the stale predator odors, were used in an attempt to find as many effective stimuli as possible in order to study the relationship between HR and $\dot{V}_{O_2}$ in alert wapiti, which was the main objective of the larger study. They were not necessarily well suited to demonstrate whether or not prey react to predator odors. The extract of cougar feces produced no detectable effect, whereas whole cougar

feces were very effective. It is not clear if this is due to a difference in odor intensity (see above) or to the absence of effective volatiles in E1. As for PDT, it is a synthetic compound that has proven effective with many small prey species, but it is from a carnivore that poses no threat to wapiti. Similarly, the stale predator odors probably lost essential volatiles (Dixon et al., 1987).

Thus, we felt justified to conduct another comparison, restricted to biologically irrelevant novel odors and fresh predator odors, to assess if the reaction to predator odors was merely due to novelty. Fresh predator odors produced a higher proportion of significant tachycardias, and on average, larger tachycardias, than the novel odors, although there was no difference between these two classes in terms of their effect on $\dot{V}_{O_2}$. Even though fresh predator odors were more effective as a group, some of these odors were ineffective. In the case of coyote feces, which were collected fresh in the study area a few weeks before being used in trials, this was especially surprising, coyotes being a potential predator of wapiti calves in this area. This lack of response could be due to the fact that we were able to conduct only three trials with this stimulus, one of them being with Blue, who did not react to any smell. In addition, the quantity used was less than that of the other trials involving predator feces (Table 2). The other fresh predator odor, the dead coyote, may not have been representative of the smell of a live coyote.

Predator Odors in Assessing Predation Risk

The increases observed in HR (2.85–10.23 bpm) or $\dot{V}_{O_2}$ (0.29–0.93 liters/min) of wapiti in response to predator odors were very mild. By comparison, 1-min exposures to fawn distress calls or close proximity to human beings frequently resulted in HR increases of 25–50 bpm, and $\dot{V}_{O_2}$ increases of 0.5–1.7 liters/min (Chabot, 1992). It has been shown that the size of HR responses is indicative of perceived danger and the level of stress induced in animals (Tatoyan and Cherkovich, 1972; MacArthur et al., 1979, 1982; Ball and Amlaner, 1980; Harlow et al., 1987a,b). The small HR responses to odors we observed in this study indicate low arousal levels. This suggests that the subjects were aware of the stimulus but did not consider it an immediate threat, perhaps because coytes were the only natural predator in the study area or because the odors were considered irrelevant in the experimental context (Albone et al., 1986). Even though rats and geckos have shown typical antipredator behavior when exposed to predator odors (Vernet-Maury, 1980; Vernet-Maury et al., 1984; Dial, 1990; Weldon, 1990), there is no evidence that ungulates react that strongly to predator odors alone. Pfister et al. (1990), for instance, reported that with sheep (*Ovis aries*) and cattle (*Bos taurus*), predator odors in feed bins did not reduce the number of approaches or head entries in the bins, but resulted in animals spending less time in proximity of predator-contaminated bias. Müller-

Schwarze (1972) and Sullivan et al. (1985b) also noted that black-tailed deer (*Odocoileus hemionus columbianus*) closely investigated predator odors instead of immediately moving away.

Such findings and the small HR responses obtained here are more compatible with the hypothesis that prey species, as part of their antipredator strategy, assess the risk of predator encounters in various parts of their habitat, and information about predator density can be obtained from predator feces or urine (Lima and Dill, 1990). Such assessment could then be used to make decisions about where and when to feed. The work of Sullivan et al. (1990b) with gophers and of Pfister et al. (1990) with sheep and cattle are most relevant here, because they demonstrate that some prey indeed avoid areas because of predator odors or spend less time and feed less in areas contaminated with predator odors. For bighorn sheep, HR increased as distance to a road decreased, when sheep traveled in timber where visibility was reduced, and when distance to escape terrain increased, suggesting that they perceive risk levels associated with their location or their own behavior. Many other species have been shown to alter their behavior according to perceived predation risk (for a review, see Lima and Dill, 1990).

In this context, care should be taken when making interspecific comparisons. Not all species can make use of predator feces and urine to assess predation risk. Species that normally live where predator density is high probably find such cues of limited value, because they would lead to a large number of false alarms or to the avoidance of large proportions of available habitat. When predators are abundant and the habitat is open, other cues should be used. Walther (1984, pp. 366–367) has observed that African ungulates pay little attention to the many predators around them, so proximity to predators does not always elicit fear or even avoidance. Prey may even be able to discriminate hungry from satiated predators, as demonstrated in guppies (Licht, 1989). For other species, detection avoidance is more important than evasion from predators. For example, in the absence of cover, Townsend's voles (*Microtus townsendii*) preferred to feed on the side of their enclosure without repellents, but when cover was present, voles preferred to feed on the side with cover, regardless of whether or not repellents were present (Merkens et al., 1991).

On the other hand, chemical cues associated with the close proximity of a predator should have effects similar to the sighting of a predator at close range or to the alarm signals of conspecifics. Bighorn sheep show large tachycardias upon sighting dogs or coyotes (MacArthur et al., 1979). While we have no measure of HR when wapiti located a coyote, the study animals were extremely aggressive every time they sighted dogs, and wapiti are known to mob coyotes (Geist, 1982). Thus it is surprising that the body odor of a coyote had no effect on the HR of the five wapiti exposed to it, although it did result in increased oxygen consumption in two of them. Only one coyote was available as a test stimulus. It had been dead for over a month and kept in a freezer containing

other wildlife carcasses, so it may not have been representative of the odor of a live coyote. Additional experiments with live predators, controlling for the kind of information the prey could use (odor, sounds, sight, alone and in combination) and, depending on prey species, with alarm calls or alarm pheromones, would be useful.

Habituation

When the same odor was presented to a wapiti a second time, on average it resulted in a smaller ΔHR and $\Delta\dot{V}_{O_2}$, although the latter did not decrease significantly. Fewer trials were effective on second presentation, but again, this was not significant. Our results suggest that habituation can take place even for biologically relevant stimuli in the absence of feedback. Thompson et al. (1968a) and Espmark and Langvatn (1985) made similar observations with starlings and red deer calves. We had some indication that longer delays might not cause habituation: three of 19 cases had stronger responses on second trial than on the original trial, and all three had delays $\geq$ 173 hr. However, one case was significant on first presentation but not on a second test 215 hr later. Sample sizes were very small for repeated trials, and there was one confounding variable: most of the trials that were effective on the first presentation but not on the second presentation used whole feces. For many of these we reused the same odor sample approximately 24 hr later. Dixon et al. (1987) have shown that new compounds are formed when feces are allowed to age even for a few hours. Therefore, new experiments are required to clarify the question of habituation. Stimulus intensity and quality should be the same on all repeated trials, and a greater range of delays between trials should be used.

HR Telemetry in Future Research on Predator Odors

It was unanticipated that some odors would increase $\dot{V}_{O_2}$ but not HR, and that overall, $\dot{V}_{O_2}$ would be as sensitive a measure of response to predator odors as HR. Many studies showed stronger HR than $\dot{V}_{O_2}$ responses to various stimuli in humans (Blix et al., 1974; Turner and Carroll, 1985; Allen et al., 1986; Sherwood et al., 1986), but this contradiction may be more apparent than real: those studies emphasized that humans react to alertness with exaggerated tachycardias relative to concomitant metabolic demand. However, $\dot{V}_{O_2}$ increased too, but this was not tested statistically. Yet our results confirm that the two variables do not always vary in unison, a difficulty if HR is to be used as an index of $\dot{V}_{O_2}$ (Floyd, 1987). Again, this is partly an artifact: Chabot (1992) has shown that there is a good relationship between ΔHR and $\Delta\dot{V}_{O_2}$ when wapiti hinds became alert, but with small HR and $\dot{V}_{O_2}$ responses, like those found in this study, variability around the regression line can result in apparent disagreement between the two variables.

Even if it proved sensitive here, it is unlikely that $\dot{V}_{O_2}$ will become a widely used index of reaction to stimuli, since it is more difficult to measure in a natural context. Albone et al. (1986) have stressed that mammals do not generally respond to chemical stimuli in isolation, but integrate them with other stimuli. Similarly, Merkens et al. (1991) demonstrated the importance of context for the reaction of mammals to predator odors. The ease with which responses to predator odors can be detected with HR telemetry should make it easier to study the reaction of free-ranging animals to predator odors, be they naturally occurring or provided by experimenters.

Because many researchers used a reduction in feeding levels to demonstrate a reaction to predator odors, it would be relevant to measure the HR responses to predator odors in at least one species that is known to reduce its feeding levels when exposed to predator odors. This should allow the comparison of the two methods and perhaps demonstrate that reduced palatability was not the explanation for reduced feeding levels. Such an experiment should compare HR responses to predator odors, to malodorant nonpredator odors, and to substances known to reduce palatability of food and be conducted both in foraging and nonforaging situations.

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ATOMIC MODEL OF THE RECOGNITION SITE OF THE AMERICAN COCKROACH PHEROMONE RECEPTOR

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Abstract—All the minimum-energy conformations (MECs) of two sex pheromones of the American cockroach, *Periplaneta americana*, and their 11 structural analogs (seven agonists, two antagonists, and two inactive compounds) were calculated using the molecular mechanics method. The MECs of the analogs were compared with the most populated MECs of the pheromones. The MECs most common in all the ligands were assumed to represent the bioactive conformations. An atomic model complementary to the bioactive conformation of one of the pheromones, periplanone B, was constructed. The model incorporates five groups capable of forming H bonds with oxygen atoms of the ligands and a set of hydrogen atoms contributing to nonbonded interactions with the ligands. Using this model, the energies of ligand–receptor complexes were calculated. For a group of mimics, the activities predicted from the calculated energies of ligand-receptor interactions were in reasonable agreement with the experimentally observed activities. The sites of the receptor model essential for the receptor activation were specified.

Key Words—Periplanone B, molecular mechanics, pheromone antagonists, pheromone agonists, bioactive conformation.

INTRODUCTION

Pheromone receptors are membrane proteins that belong to the superfamily of G-protein-coupled receptors (Buck and Axel, 1991). As the three-dimensional

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structure of the receptors remains unknown, it is the analysis of structure–activity and conformation–activity relationships of the ligands that may clarify their structural peculiarities important for ligand-receptor recognition.

Periplanone A (PA) and periplanone B (PB) (Figure 1) are the most active components of the sex pheromone of the American cockroach. In the male antennae, there are two types of receptor cells (A and B) sensitive to the sex pheromone (Sass, 1983). Recordings of the single sensilla impulse activity (Hartmann, 1988) demonstrated that the A cells are activated by PA only, while the B cells may be activated by either PA or PB.

Several studies of the structure–activity relationships of periplanone analogs have been published (see Figure 1 and Table 1). According to the behavioral experiments, seven analogs (III–IX) are mimics, compounds X and XI are antagonists, and compounds XII and XIII are inactive. Antagonist XI inhibited activity of PA, while antagonist X inhibited activity of both PA and PB. All the

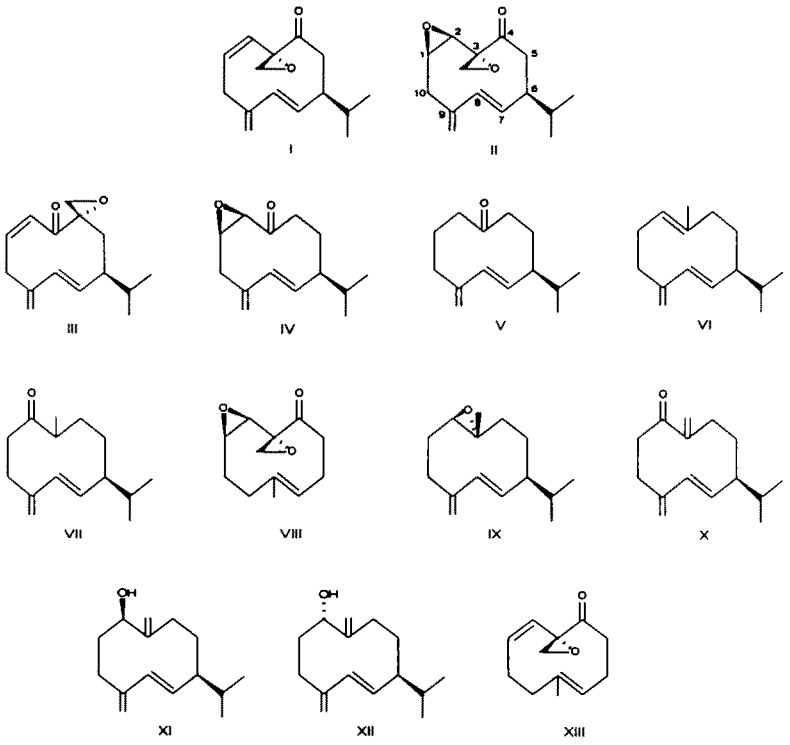

FIG. 1. The chemical structures of the sex pheromones PA (I), PB (II), their agonists (III–IX), antagonists (X and XI), and inactive compounds (XII and XIII).

TABLE 1. ACTIVITY (THRESHOLD DOSE) OF AMERICAN COCKROACH SEX PHEROMONES AND ANALOGS AS DETERMINED IN BEHAVIORAL AND ELECTROPHYSIOLOGICAL EXPERIMENTS

Compound	Activity (μg)[a]			Reference
	A cell	B cell	Behavioral reaction	
Agonists				
I (PA)	10^{-7}	10^{-5}	10^{-5}	Boeckh et al. (1990); Okada et al. (1990)
II (PB)		10^{-7}	10^{-7}	Boeckh et al. (1990); Still (1979)
III			10^{-4}	Okada et al. (1990)
IV			0.1	Okada et al. (1990)
V			1	Okada et al. (1990)
VI (GD)	1	100	5	Hartmann et al. (1988); Manabe and Nishino (1985)
VII			20	Nishino et al. (1989)
VIII			10^{-3}	Mori et al. (1990a)
IX			20	Nishino et al. (1989)
Antagonists				
X	80	80		Nishino et al. (1989)
XI	80			Nishino et al. (1989)
Inactive compounds				
XII				Nishino et al. (1989)
XIII				Okada et al. (1991)

[a]The activity of agonists is the threshold dose causing the impulse electrical response of receptor cell A or B or the behavioral reaction of a male. The activity of antagonists is the dose inhibiting the behavioral reaction of a male when mixed with 2×10^{-4} μg of periplanone A or B.

mimics and antagonist XI evoked a significant electrical response in the male antenna. In single-sensilla recordings (Hartmann, 1988), the mimic VI, germacrene D (GD), exhibited stronger activity at the A cells than at the B cells. Thus, although the above compounds are similar in chemical structure (see Figure 1), they demonstrate essentially different biological activity. To explain this, an analysis of three-dimensional structures of the ligands is desirable.

Three-dimensional structures of compounds I–III were determined by X-ray analysis (Mori et al., 1990b). However, each compound is flexible and exists as a mixture of different conformers. If these conformers are close in energy, all of them would be noticeably populated. In such a case, the biologically active conformation of a ligand would not necessarily correspond to the crystalline one. Shimazaki et al. (1991, 1992) calculated minimum-energy conformations (MECs) of the compounds II, IV–VI, and VIII by the molecular mechanics and quantum chemistry methods, suggesting the low-energy MECs to represent the bioactive conformations. Most of the mimics shown in Table 1

exhibit activity essentially lower than that of the natural pheromones. The low activity of the mimics may be due to a low population (high energy) of their bioactive conformation. Therefore, the high-energy MECs should also be considered as candidates for the bioactive conformations.

In the present study, the theoretical conformational analyses of PA, PB, and their analogs have been carried out. The bioactive MEC of PB was determined and a monoatomic shell surrounding it was constructed to represent a model of the PB receptor recognition site. The energy of ligand–receptor interaction calculated for a series of PB analoges was found to correlate with their activity. The preliminary results of this study have been published (Bykhovskaya and Zhorov, 1993).

METHODS AND MATERIALS

Since compounds I–V and VIII–XIII were obtained by stereoselective synthesis (see references in Table 1), the absolute configuration of their chiral atoms is definite. The absolute configuration of atom C-3 in the active isomer of compound VII remains unknown. Therefore both 3*R* and 3*S* enantiomers of compound VII were considered.

All the MECs were calculated by energy minimization in the space of torsion and valence angles (Zhorov, 1993). The latest version of the ZMM program package for molecular mechanics calculations (Zhorov, 1981, 1982) was used. Atomic charges were calculated by the CNDO/2 method. Dielectric constant $\epsilon = 4$ was used for the calculations of electrostatic energy as recommended by Lipkind et al. (1970) for nonpolar surrounding. The hydrogen bonds were calculated using Morse potential with the minimal energy 2 kcal/mol and the optimal distances 1.8 and 2.1 Å for NH. . .O and NH. . .C_{sp2} bonds, respectively (the latter was taken into account for GD (VI) only). Initial loop-closed geometries for the 10-membered rings were generated both systematically (Go and Scheraga, 1970) and randomly.

RESULTS AND DISCUSSION

Conformational Analysis

The 10-membered ring of PB has seven MECs with energy within 3 kcal/mol of the global minimum (Figure 2, Table 2). The global minimum (MEC 1) corresponds to the structure found in X-ray study (Adams, 1979). MEC 2 has an energy of 0.1 kcal/mol above the global minimum. Interestingly, this MEC was not found in the molecular mechanics study of PB by Shimazaki et al. (1992).

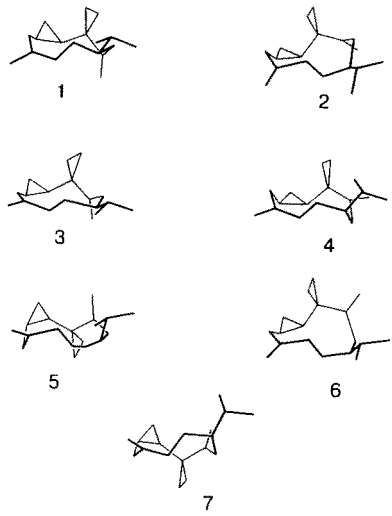

FIG. 2. MECs of PB with the energy within 3 kcal/mol of the global minimum. All the MECs are shown from the side of isopropyl and terminal methylene groups. MECs are numbered as in Tables 2–4.

TABLE 2. ENDOCYCLIC TORSIONAL ANGLES (IN DEGREES) OF PB IN CALCULATED LOWEST-ENERGY CONFORMATIONS AND X-RAY CONFORMATION

	Bond									
MEC[a]	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-1
1	−7	99	−149	96	−43	101	−174	45	74	−82
2	−22	140	−113	86	−50	−58	176	−99	−67	68
3	27	94	−153	96	−55	81	−174	126	12	−69
4	−29	143	−59	−62	63	67	−173	59	55	−75
5	4	−97	153	−99	42	−89	174	−61	−67	81
6	−14	136	−23	−76	66	−93	174	−89	50	−67
7	8	−117	127	−103	50	52	−175	111	−67	69
X-ray[b]	−2	97	−149	96	−41	110	−166	30	80	−90

[a] MEC numbers correspond to those shown in Figure 2. The orientation of isopropyl group is the same for all the compounds: both methyl groups are in *trans* position relative to bonds C-6–C-5 and C-6–C-7 (Figure 1).

[b] From Shimazaki et al. (1992).

Bioactive Conformations

The bioactive MECs of all the active ligands were assumed to have similar torsional angles of the 10-membered ring and similar disposition of the proton acceptor atoms. The selection of the bioactive MECs has been done in two stages. At the first stage, we compared the seven lowest-energy MECs of PB with all the MECs of 12 PB analogs to select the MECs with similar torsional angles. The 10-membered rings of two compounds were regarded to have a similar spatial structure if the root mean square deviation of their endocyclic torsions was less than 45° and the maximum deviation was less than 90°. Six MECs of PB turned out to have similar MECs in the analogs (Table 3). Thus the global minima of compounds I–V and XI–XIII were similar to MEC 1 of PB. The global minima of compounds VI, *R*-VII, IX, and X were similar to MEC 4 of PB. The global minimum of compound VIII was similar to MEC 2 of PB. Only MEC 3 of PB with an energy 0.8 kcal/mol above the global minimum did not have matches for the other active ligands. Therefore, the MEC 3 was excluded from the list of the candidates for the bioactive conformation.

At the second stage, we selected the MECs with similar spatial dispositions

TABLE 3. ENERGY[a] (KILOCALORIES PER MOLE) OF LOWEST ENERGY MECs IN PERIPLANONES (I, II) AND CORRESPONDING MECs IN PERIPLANONE ANALOGS

	Conformation[b]					
Compound	1	2	4	5	6	7
II (PB)	0.0	0.1	1.1	2.3	2.3	2.6
I (PA)	0.0	0.7	1.8	2.6	2.5	3.1
III	0.0	1.2	0.2	1.8	2.5	1.8
IV	0.0	0.9	0.3	1.7	1.9	1.9
V	0.0	3.0	0.4	1.7	4.9	3.8
VI (GD)	0.1	2.2	0.0	2.1	3.6	3.1
R-VII	2.1	4.3	0.0	5.0	5.2	6.1
S-VII	2.8	5.4	3.4	3.6	9.8	3.8
VIII	0.4	0.0	2.1	1.9	1.7	2.9
IX	2.1	3.7	0.0		4.7	
Antagonists						
X	0.0	2.6	0.0	1.9	4.3	3.1
XI	0.0	3.1	0.5	4.3	5.5	6.2
Inactive compounds						
XII	0.0	0.4	2.2	1.5	1.7	2.3
XIII	0.0	3.0	0.3	1.1	6.1	3.2

[a] Relative to the corresponding global minimum.

[b] The numbers of the conformations correspond to those shown in Figure 2. The orientation of the isopropyl group is same for all compounds: both methyl groups are *trans* relative to the bonds C-6–C-5 and C-6–C-7 (Figure 1).

of the proton acceptor atoms in PB and active analogs. The MECs with the similar torsional angles were superimposed by bringing together isopropyl and terminal methylene groups (Figure 3). Two given proton acceptor atoms of two given ligands were assumed to form an H bond with the same H-N group of the receptor if (1) van der Waals spheres of the atoms overlapped (the centers of the atoms were separated by less than the sum of their van der Waals radii) and (2) the overlapping atoms were not shielded from each other by interposing atoms, i.e., some part of the space common for both spheres did not overlap with any other atoms of the superimposed ligands (Figure 3).

The calculated distances between proton acceptor atoms of the superimposed ligands are given in Table 4. It shows that in MEC 2, compounds VII and X have an oxygen atom separated from the nearest oxygen of the superimposed PB by more than 2.8 Å (van der Waals diameter of the oxygen atom). In MECs 4–7, at least one mimic has a proton acceptor atom shielded by interposing atoms from any oxygen atom of the superimposed PB. Therefore, these MECs were not considered as the candidates for the bioactive conformations. Since MEC 3 was excluded at the first stage of the selection, MEC 1 remained the only candidate for the bioactive conformation. This MEC was used as a template to construct the model of the receptor.

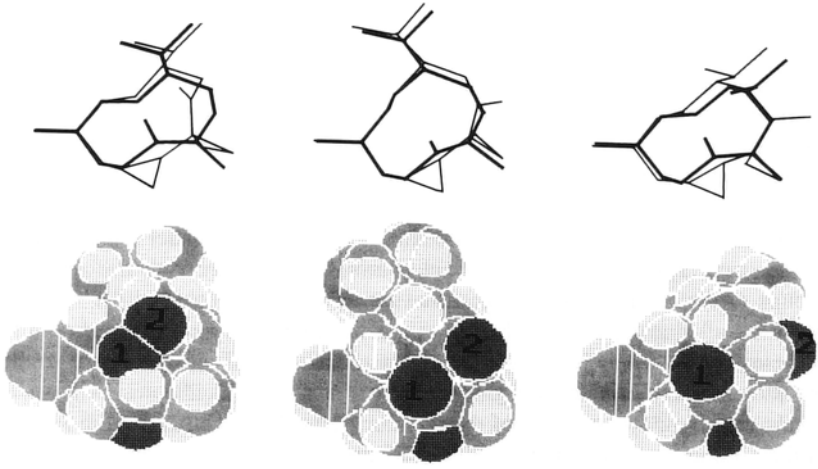

FIG. 3. Wire-frame (top) and space-filling (bottom) models of PB superimposed with compound (*R*-VII). Atom O-4 of PB is denoted by 1; oxygen atom of *R*-VII is denoted by 2. In MEC 1 (left) atoms 1 and 2 overlap (have similar spatial position). In MEC 2 (middle), the distance between atoms 1 and 2 is larger than the sum of their van der Waals radii. In MEC 3 (right) atoms 1 and 2 are separated by carbon and hydrogen atoms. the structures were visualized using the DTMM package (Oxford Electronic Publishing).

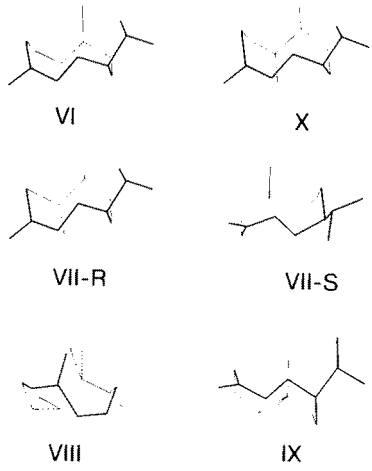

FIG. 4. The lowest-energy MECs (global minima) of compounds (VI–X). All the MECs are shown from the side of isopropyl and terminal methylene groups. The global minimum of the compound (*S*-VII) is not similar to any of the seven lowest-energy MECs of PB but it is symmetric to MEC 4.

The Receptor Model. The pheromone receptor recognition site was modeled as a monoatomic shell surrounding the bioactive conformation of periplanone B. It was constructed using the approach of Zhorov and Govyrin (1984). The shell comprises atoms and groups assumed to be involved in hydrophobic interactions and H bonds with the ligands. The surface of the receptor involved in hydrophobic interactions with the ligands should incorporate hydrogen atoms of alkyl groups and (or) aromatic residues. Since the ligands under consideration do not have aromatic groups (probable partners to interact with the aromatic groups of the receptor), we suggest that the hydrophobic areas of the receptor comprise alkyl groups only. Therefore, a set of hydrogen atoms was chosen to represent the hydrophobic areas of the recognition site. Hydrogen atoms of the receptor hydroxyl and amide groups would be natural candidates to form H bonds with the oxygen atoms of the ligands. These hydrogen atoms are similar in their ability to form H bonds. Since NH groups are common for both protein backbone and side chains (in some residues), we have chosen these groups to represent the proton donors of the receptor. Thus, the receptor model was suggested to incorporate neutral hydrogen atoms to interact with the hydrophobic groups of the ligands and NH groups to form H bonds with the ligands.

The partial charges of $-0.2e$ and $+0.1e$ (e is the proton charge) were assigned respectively for the nitrogen and hydrogen atoms of the receptor amide

TABLE 4. DISTANCES[a] (Å) BETWEEN PROTON ACCEPTOR ATOMS IN PB AND THOSE IN SUPERIMPOSED ANALOGS

Superimposed with PB		PB	Conformation					
Compound	atom[b]	atom	1	2	4	5	6	7
I	O-3	O-3	0.7	0.3	0.3	0.4	0.5	1.3
I	O-4	O-4	1.0	0.4	0.5	0.5	0.9	0.8
III	O-3	O-3	0.7	0.4	1.3	—	1.4	—
III	O-4	O-4	0.4	0.4	0.6	—	—	—
IV	O-1	O-1	0.1	0.2	0.7	1.6	0.5	1.6
IV	O-3	O-3	0.3	0.3	1.7	—	0.1	—
V	O-3	O-3	0.5	1.1	1.4	—	1.5	—
VI	(C-2=C-3)[c]	O-4	2.5	2.8	3.3	—	—	—
VI	(C-2=C-3)	O-3	—	—	—	2.0	—	2.4
R-VII	O-2	O-4	1.7	2.9	—	1.7	—	2.7
S-VII	O-2	O-4	1.6	3.5	—	1.7	—	2.3
IX	O-2	O-4	2.8	2.8	—		—	
X	O-2	O-4	2.0	3.6	—	2.1	—	2.8

[a]The dash means that the pair of atoms was separated by one or several interposing atoms of the ligand (see text and Figure 3, right).
[b]The number of an oxygen atom corresponds to the number of the carbon atom the oxygen is bound to (Figure 1).
[c]The midpoint of the bond C-2=C-3 represents the location of the proton acceptor in GD.

groups. An NH group of the receptor was placed against each lone pair (LP) of PB, the atoms N-H. . .LP-O forming a straight line with the distance LP. . .H equal to 1.8 Å. If two NH groups of the receptor overlapped, they were replaced by a single one positioned between them. The positions and the orientations of the receptor NH groups were then optimized by energy minimization with PB kept rigid within the shell.

A "cup" of seven uncharged hydrogen atoms of the receptor shell was placed against each hydrogen atom of the isopropyl, terminal methylene, and epoxy groups of PB, the distance between any pair of the eight hydrogen atoms being 2.4 Å (van der Waals diameter of the hydrogen atom). One atom of the cup was positioned at the extension of a given C-H bond of PB, six others being in the plane normal to the given C-H bond. All hydrogen atoms of the receptor separated from any atom of PB or from any polar group of the shell or from a couple of uncharged hydrogen atoms of the shell by less than the sum of the corresponding van der Waals radii were removed. Then each couple of the uncharged atoms of the shell separated by less than 2.4 Å was replaced by a single atom positioned exactly between them. The model thus constructed incor-

porated five NH groups forming six H bonds with oxygen atoms of PB (one NH group turned out to form a bifurcate H bond with two oxygen atoms of PB) and 26 hydrogen atoms surrounding the isopropyl, terminal methylene, and epoxy groups of PB (Figure 5).

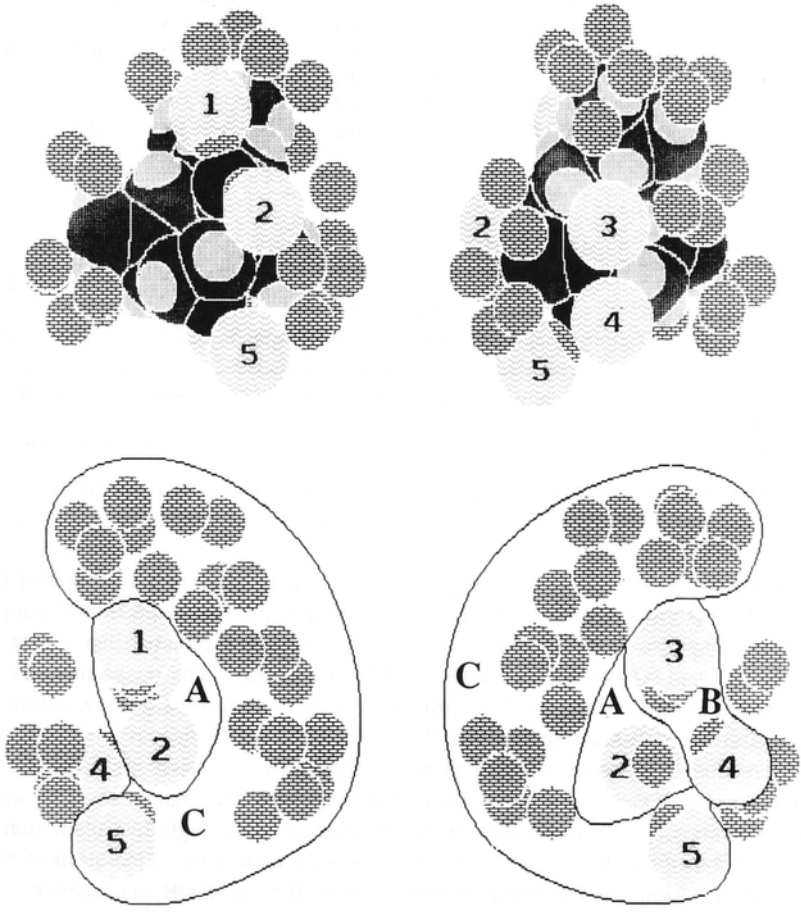

FIG. 5. The space-filled model of PB receptor (bottom) and its complex with PB (top) viewed from the opposite sides. PB oxygens, receptor hydrogens, and receptor nitrogens are outlined with dark, brick, and wave patterns, respectively. Receptor NH groups are number 1 through 5. The solid lines (bottom) separate receptor sites A, B, and C. Site A incorporates NH groups 1 and 2; site B, NH groups 3 and 4; site C, 21 hydrogen atom and NH group 5.

Ligand–Receptor Interactions. Using the constructed atomic model of PB receptor, we predicted the energy of the receptor interaction with different ligands. The bioactive MEC of each periplanone analog was superimposed with PB bioactive MEC inside the shell. Then PB was removed and the ligand conformation and orientation were optimized by energy minimization within the rigid receptor. The obtained energy of receptor–ligand complex (E_{rlc}) represented the sum of receptor–ligand interactions (E_{rli}) and intramolecular interactions of the ligand in the bioactive conformation E_{lbc}):

$$E_{rlc} = E_{rli} + E_{lbc}$$

With reference to the ligands' global minimum (E_{lgl}), the energy of the receptor–ligand complex (ΔE_{rlc}) and the energy of the ligand bioactive conformation (ΔE_{lbc}) were calculated as:

$$\Delta E_{rlc} = E_{rlc} - E_{lgl}$$

$$\Delta E_{lbc} = E_{lbc} - E_{lgl}$$

The activity of the jth ligand relative to that of PB was predicted to be

$$AP^j = \exp\left[-(\Delta E^j_{rlc} - \Delta E^{PB}_{rlc})/RT\right]$$

where R is the universal gas constant and T is temperature. The predicted relative activities were compared with the experimental ones

$$AE^j = TD^{PB}/TD^j$$

where TD^j and TD^{PB} are threshold doses of the jth ligand and PB, respectively.

The results (Table 5) demonstrate a reasonable correlation between the predicted and experimental activities for mimics I, IV, V, VI, and VIII. The experimental activities of mimics III, VII, and IX are greater than the predicted ones. A possible explanation is that these mimics cause the behavioral reaction of the cockroach via activating the A receptor. Calculations demonstrate that only the R isomer of compound VII should be active. This prediction may be tested experimentally.

According to the calculations, compound XIII should be more active than compounds VI, VII, and IX, whereas experiments show compound XIII to be inactive. This may be explained in view of the hypothesis that a pheromone receptor is activated when several of its functional sites interact simultaneously with a ligand (Kafka and Neuwirth, 1975, Kikuchi and Ogura, 1976). We subdivided the receptor model into three functional sites (Figure 5): site A, incorporating NH groups 1 and 2 (they H bond to atom O-4 of PB); site B, incorporating NH groups 3 and 4 (the NH group 3 H bonds to atom O-3 of PB and the NH group 4 forms a bifurcate H bond with O-1 and O-3 of PB); site C, incorporating NH group 5 (it H bonds to atom O-1 of PB); and 21 hydrogen

TABLE 5. ENERGY (KILOCALORIES PER MOLE) OF LIGAND–RECEPTOR INTERACTIONS PREDICTED AND EXPERIMENTAL ACTIVITIES OF LIGANDS[a]

Compound	ΔE_{lbc}	E_{rli}	ΔE_{rlc}	$-\log_{10}$AP	$-\log_{10}$A		Receptor sites involved in binding
					B cell	Behavior	
II	0.0	−27.8	−27.8	1.0	1.0	1.0	A, B, C
I	0.0	−24.1	−24.1	2.7	2.0	2.0	A, B, C
III	0.0	−20.4	−20.4	5.4		3.0	A, B, C
IV	0.0	−20.1	−20.1	5.6		6.0	B, C
V	0.0	−18.1	−18.1	7.0		7.0	B, C
VI	0.1	−16.7	−16.6	8.1	9.0	7.6	A, C
R-VII	2.1	−14.8	−12.7	10.9		8.3	A, C
S-VII	2.8	−9.7	−6.9	15.1			A, C
VIII	0.4	−22.4	−22.0	4.2		4.0	A, B, C
IX	2.1	−9.6	−7.5	14.7		8.3	A, C
Antagonists							
X	0.0	−16.0	−16.0	8.5			A, C
XI	0.0	−11.8	−11.8	11.6			C
Inactive compounds							
XII	0.0	−13.3	−13.3	10.5			C
XIII	0.0	−17.7	−17.7	7.3			A, B

[a] ΔE_{lbc} is the energy of the ligand bioactive conformation above the global minimum; E_{rli} is the energy of interactions of the ligand's bioactive conformation with the receptor model; ΔE_{rlc} is the energy of ligand-receptor complex relative to the ligand's global minimum; AP is the predicted activity of the ligand relative to PB; AE is the experimental activity of the ligand relative to PB (see text).

atoms (they interact with alkyl fragments of the ligands, in particular, with the terminal methylene and isopropyl groups of PB). We assume that to activate the B receptor, a ligand should interact with at least two sites: either C and B or C and A.

All the agonists as well as PB atagonist X meet the above requirement (Table 5). Inactive compound XIII lacks the isoproyl, terminal methylene, and spiro epoxy groups, and, hence, cannot interact with the receptor site C. Inactive compound XII and PA-specific antagonist XI interact with site C and, in addition, have oxygen atoms which may H bond to NH groups of the site A. However, the calculated energies of these H bonds were less than 1 kcal/mol, whereas the H-bond energies for all the active compounds were greater than 2.5 kcal/mol. This means that the probability of the compounds XI and XII forming H bonds with the receptor site A is 10 times less than that for the active compounds.

In experiments by Nishino et al. (1989), compound IX evoked an electrical response by the male antenna competing only with PA, whereas the available

quantity of compound X was not enough for electrophysiological experiments. Our model predicts compound X to be the PB agonist, while experiments show it to be a PB antagonist. A possible reason is the compound X blocks the cockroach behavioral reaction through nonpheromone receptor cells. This prediction may be tested in electrophysiological experiments.

Thus, the proposed model of the PB receptor predicts the energy of ligand–receptor interactions, in agreement with the experimental activity for those five agonists that interact with receptor sites B and C (Figure 5). The agonists that interact with sites A and C have experimental activities higher than the predicted ones (Table 5). One of the agonists, germacrene D (VI), is known to activate PA receptor. The others may also activate the PA receptor. The simultaneous interaction of a ligand with sites B and C or A and C is suggested to be necessary for PB receptor activation. Inactive compounds XI and XII, which are similar in structure to active compounds VII and X, as well as inactive compound XIII, which is similar to active compounds I and VIII, do not satisfy this requirement.

CONCLUSION

In this work, we have carried out conformational analysis of a series of periplanone B and its 12 analogs. Using a set of energetic and geometric criteria, we determined the bioactive conformation of the ligands of the periplanone B receptor. The bioactive conformation of the most active ligand, periplanone B, was used as a template to construct an atomic model of the receptor recognition site. The atomic model enabled us to calculate energies of ligand–receptor interactions and, hence, to predict activities of the ligands. A reasonable agreement between the predicted and experimental activities was demonstrated for the majority of the mimics. The exception was found in three mimics whose activities in behavioral experiments were higher than those predicted. A possible explanation of the exception is the interaction of the three compounds not only with the periplanone B receptor but also with the periplanone A receptor (another pheromone receptor in the male sensilla). Distinct functional sites in the receptor model have been specified, suggesting that simultaneous interaction of a ligand with at least two functional sites is necessary to activate the receptor. Inactivity of three close structural analogs of periplanone B was explained by their inability to interest simultaneously with two sites of the receptor.

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PREDATOR-INDUCED DIEL VERTICAL MIGRATION IN *Daphnia*: ENRICHMENT AND PRELIMINARY CHEMICAL CHARACTERIZATION OF A KAIROMONE EXUDED BY FISH

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Abstract—Planktivorous fish release a cue of an unknown chemical nature into the water that induces diel vertical migration in *Daphnia* as predator avoidance response. We used a bioassay to obtain information about the chemical nature of the kairomone. We present a method to enrich the cue from holding water of fish by sorbent extraction. The kairomone exuded by *Leucaspius delineatus* can be characterized as a nonolefinic low-molecular-weight anion of intermediate lipophilicity. The presence of amino groups in the molecule can be excluded, whereas hydroxy groups are essential for activity. Separation by HPLC yielded only one active fraction. The kairomones released by *Carassius carassius* and *Rutilus rutilus* showed the same chemical characteristics, suggesting that the kairomones from different species of fish, sensed by *Daphia*, are very similar, if not identical.

Key Words—Behavior, chemical communication, Cladocera, *Daphnia*, diel vertical migration, kairomone, predator avoidance, zooplankton.

INTRODUCTION

The phenomenon of diel vertical migration (DVM) is a common behavior both in marine and in freshwater zooplankton. During the day, the zooplankton stay in the deep layer of the water column and reappear at the surface during the

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night. According to the predator-avoidance hypothesis, this behavior reduces the risk of daytime predation by visually orienting predators, mostly fish (Zaret and Suffern, 1976; Lampert, 1989). It has been shown that DVM can be induced by predator-borne chemical cues in a number of zooplankton species (Dodson, 1988; Neill, 1990; Dawidowicz et al., 1990). *Daphnia* (Cladocera), the most important grazer of phytoplankton in temperate lakes, is among the species that respond to fish-exuded kairomones (Dodson, 1988).

The benefit of DVM behavior, reduced predation risk, is traded off with metabolic costs due to low temperature and food levels during the daytime stay in the hypolimnion (Dawidowicz and Loose, 1992). As the abundance of planktivorous fish in the pelagic zone fluctuates during both the course of one season and between seasons, a link between the strength of DVM and the actual risk of predation should be highly adaptive (Pijanowska, 1993). A tightly coupled response of zooplankton towards increasing and decreasing abundance of planktivorous fish has indeed been observed both in the field (Ringelberg et al., 1991) and experimentally (Bollens and Frost, 1991; Loose, 1993; Loose and Dawidowicz, 1994).

The kairomone represents this link between the presence of fish and the behavior of *Daphnia*, and thus its reliability is crucial in order to avoid maladaptive behavior in *Daphnia* (Fagen, 1987). An important feature of the kairomone's reliability is its behavior in the water, which is determined by its chemical nature. Recently, a bioassay has been described that allowed a first characterization of the kairomone released by *Leucaspius delineatus* (Loose et al., 1993). The cue was characterized as low molecular weight, water-soluble, and stable over broad ranges of temperature and pH. Here we present further chemical characterization of the kairomone of *L. delineatus* and a method to enrich the cue from holding water of fish. Both aspects are an important step towards the identification of the kairomone. The kairomones from three cyprinid fish species are compared chemically and by HPLC.

METHOD AND MATERIALS

Bioassay. The characterization of the kairomone was performed by using a bioassay based upon the DVM behavior of small cohorts of *Daphnia*. The standardized experimental design has been shown to be a reliable indicator for the assessment of the kairomone (Loose et al., 1993). A clone of *Daphnia magna* was used as test animals. It was isolated from Grosser Binnensee (northern Germany), where it coexists with fish (Lampert, 1991). Five 4-day-old animals were used per treatment. They were incubated in Perspex tubes (1 m length, 200 ml volume) and placed in a water bath with a defined temperature stratification (20°C at the top, 10°C at the bottom). The tubes were illuminated

from the top (16:8 hr day–night cycle), and *Scenedesmus acutus* was added as food (2 mg C/liter initial concentration). For further details see Loose et al. (1993). The mean day depth of the animals on the third day of incubation was taken as the measure of activity. Mean depths deeper than 60 cm (animals in the cold hypolimnion) indicated the presence of kairomone, whereas a mean depth above 30 cm indicated the absence of the cue.

Production and Enrichment of Kairomone. Artificial medium (Tollrian, 1993) was used for control treatments. Water containing the kairomone was prepared as follows: two to eight adult fish of three different cyprinid fish species (*Leucaspius delineatus*, *Carassius carassius*, *Rutilus rutilus*), were fed with frozen chironomids once a week and kept for 24 hr in 8 liters of artificial medium. Then the water was filtered through a 0.45-μm filter. Filtered holding water was diluted 1:10 with artificial medium prior to biotest (fish water). Twenty milliliters of fish holding water were used for further treatment and eventually diluted with artificial medium prior to biotesting. For the no-fish treatments (control), we used artificial medium, which was processed in a similar way as the holding water of fish.

The kairomone was enriched from holding water by solid-phase extraction (SPE). We tested the efficiency of nonpolar C18, C8, and phenyl-SPE cartridges (500 mg, Analytichem Int.) for the enrichment of the kairomone from holding water of fish. The cartridges were preconditioned with methanol and ultrapure water, 10 ml each, prior to adding the sample. The pH of the sample was adjusted to 7.0 with 2 M HCl. Methanol was added to achieve a 1% concentration, and the resultant solution was passed through the cartridge. The loaded cartridge was washed with 5 ml of ultrapure water prior to elution of the isolate with 10 ml of methanol. Both the eluate and the eluent were evaporated to dryness in order to remove organic solvents, and the residue was resuspended in artificial medium and tested for biological activity.

Chemical Characterization of Kairomone. Polar sorbents, strong and weak anion-exchange cartridges (SAX and DEA-silica bonded sorbents), and strong cation-exchange cartridges (PRS) were washed with 5 ml of ultrapure water. Then C18-SPE-enriched kairomone from 20 ml of holding water was resuspended in 20 ml ultrapure water; the pH was adjusted to 7.5, 5.5, or 2.5 with 0.1 M HCl; and the sample was passed through the ion exchanger. The eluate was collected. For elution of ionically bound compounds, 10 ml of a NaCl solution (0.5 M and 1 M) were applied to the ion-exchange cartridge. Both eluate and eluent were diluted to a volume of 50 ml with ultrapure water and neutralized with 0.1 M HCl, or 0.1 M NaOH, respectively. Salts and contaminants, possibly toxic to *Daphnia*, were subsequently removed by reextracting the kairomone with C18-SPE.

We used base-catalyzed acetylation similar to Watts and Kekwick (1974). An aliquot of C18-SPE-enriched kairomone was gently evaporated to dryness.

Then 800 μl of dried pyridine and 200 μl of acetic anhydride were added and allowed to react for 6 hr at 60°C. The reaction was stopped by evaporation to dryness under nitrogen. The sample was resuspended in 1 ml of methanol, diluted with ultrapure water to a volume of 100 ml, and extracted by C18-SPE using the eluent for the bioassay. In order to make sure that the kairomone was inactivated but not destroyed by acetylation, reversibility of the derivatization was checked by alkaline saponification. The acetylated cue was evaporated to dryness, resuspended in 1 ml of 1 M NaOH, and incubated for 45 min at 80°C. Then the solution was diluted with ultrapure water to a volume of 50 ml, neutralized, and salts were removed by reextraction of the kairomone by C18-SPE, using the eluent (methanol) for the biotest.

Derivatization of primary amino groups was performed according to Münster et al. (1992). First 50 mg of *o*-phthaldialdehyde (OPA) were dissolved in 400 μl methanol, then 3 ml of borate buffer, pH 9.2 (Merck), and 50 μl of mercaptoethanol were added, and the reagent was stored for two days at 4°C. C18-SPE enriched kairomone from 20 ml holding water was evaporated to dryness and resuspended in 100 μl of the above-mentioned borate buffer. Fifty microliters of the reagent were added and allowed to react for 10 min. Subsequently, the sample was diluted to a volume of 50 ml with ultrapure water, neutralized, and ions were removed by reextraction of the kairomone by C18-SPE. In a different approach the dried sample was resuspended in 1 ml of 25 mM $NaHCO_3$/NaOH buffer, pH 9.0, and 30 μl of a fluorescamine solution (10 mg/100 μl acetone) were added (Parejko and Dodson, 1990). The mixture was allowed to react for 15 min prior to reextaction by C18-SPE.

For derivatization of primary and secondary amino groups according to Carpino and Grace (1972), kairomone enriched from 20 ml holding water by C18-SPE was resuspended in 180 μl of 0.13 M borate buffer, and 30 μl of a solution of 10 nmol 9-fluorenylmethyl-chloroformate (FMOC)/μl acetonitrile were added. After mixing for 2 min, the solution was diluted to a volume of 50 ml with ultrapure water, neutralized, and subjected to C18-SPE.

For catalyzed reduction, an aliquot of enriched kairomone was evaporated to dryness and resuspended in 2 ml of methanol. For platinum-catalyzed reduction with hydrogen, 100 mg of PtO_2 was added, and the solution was stirred for 6 hr under hydrogen. Reduction with $NaBH_4$ was performed by adding 100 mg of the hydride to the resuspended sample, which was then gently stirred for 6 hr in a sealed microvial. After reduction with either Pt/H_2 or $NaBH_4$, the catalyst was removed by centrifugation; the supernatant was evaporated to dryness and resuspended in ultrapure water.

Kairomone enriched by C18-SPE from 20 ml of holding water was subjected to reversed-phase HPLC on a 250-mm × 4.6-mm column packed with Biosil (C18, 5 μm, Biorad) by a linear gradient of ultrapure water and methanol (0 min: 0%; 3 min: 0%; 28 min: 100%; 36 min: 100% methanol) at a flow

rate of 1 mL/min. Fractions of 4 ml were collected, evaporated, and tested for biological activity.

RESULTS

Lipophilic Nature of Kairomone. Aliquots of holding water from *Leucaspius delineatus* were adjusted to pH values ranging from 0 to 12 with equal increments of 1 pH unit and extracted with diethyl ether. In all treatments activity remained in the aqueous layer, indicating low lipophilicity of the cue at any pH. Despite its low lipophilicity, the activity could be enriched quantitatively from water by lipophilic sorbents (Table 1). There was complete removal of activity by nonpolar sorbents and full recovery from the sorbents could be achieved by eluting with methanol. The phenyl cartridge, which more selectively interacts with aromatic molecules, showed retention abilities similar to those of the C18 and the C8 cartridges, so that an aromatic chemical nature of the cue can not be excluded. Subsequently we used C18 cartridges as the standard method to enrich the DVM-inducing activity from holding water of fish.

Interactions with Ion Exchange Resins. The hydrophilicity of the kairomone exuded from *Leucaspius delineatus* might be due to polar ionic or nonionic groups. In order to test for ionic groups, enriched activity from *L. delineatus* was passed through different ion exchange cartridges at different pH values. The

TABLE 1. RETENTION OF *Leucaspius delineatus* KAIROMONE BY DIFFERENT NONPOLAR SOLID-PHASE CARTRIDGES[a]

Treatment	Mean day depth (cm ± 95% CI)	
	Fish	Control
C18-SPE		
Eluate	3 ± 2	
Eluent	89 ± 4	10 ± 12
C8-SPE		
Eluate	16 ± 5	
Eluent	81 ± 21	14 ± 5
Phenyl-SPE		
Eluate	6 ± 3	
Eluent	80 ± 17	16 ± 7

[a] Eluate: fraction that passed the cartridge unretained. Eluent: fraction desorbed with methanol. For controls 'non-fish' water was used. Maximum mean day depth (95 cm) indicates maximum biological activity ($N = 5$).

TABLE 2. INTERACTION OF *Leucaspius delineatus* KAIROMONE WITH STRONG CATION AND WEAK ANION EXCHANGE RESINS[a]

Treatment	pH	Mean day depth (cm ± 95% CI)
Cation exchanger		
Fish	2.5	80 ± 12
Fish	5.5	73 ± 36
Fish	7.5	67 ± 30
Control	5.5	19 ± 7
Anion exchanger		
Fish	2.5	8 ± 4
Fish	5.5	5 ± 7
Fish	7.5	5 ± 10
Control	5.5	11 ± 9

[a]Only activity of the unretained fraction is reported. C18-SPE-enriched kairomone was used for fish treatments, nonfish water was used for control treatments. Maximum mean day depth (95 cm) indicates maximum biological activity.

kairomone was not retained by the cation-exchange resin at acidic or neutral pH values (Table 2). Hence no cationic groups are present in the cue. The cue was removed by a weak anion-exchange resin (Table 2). The pH values in the 2.5–7.5 range did not influence the interaction with the weak anion exchanger (Table 2), which indicates that it is not a carboxyl group that makes the kairomone an anion.

In order to investigate the nature of the interactions of the kairomone with the strong anion exchanger, an activity-loaded strong anion exchanger was sequentially developed with methanol and increasing salinity (Table 3). Lipophilic adsorption of the kairomone to the polypropylene wall of the cartridge would have resulted in an active methanol fraction, but only the 0.5 M NaCl eluate was active, indicating that the retention of the cue was due to its anionic character.

Chemical Derivatization. Characterization of functional groups essential for activity was attempted by chemical derivatization prior to biotesting. C18-SPE-enriched kairomone from *Leucaspius delineatus* was not destroyed by saponification (Table 4), which excludes esters as an essential constituent of the biological activity. Activity was inactivated by base-catalyzed acetylation (Table 4), but full biological activity was recovered after subsequent saponification. Thus the cue was not damaged by the chemical procedure itself, but a functional group that could be acetylated was essential for biological activity. To gain further information about the functional group, agents that more specifically

TABLE 3. ELUTION OF C18-SPE-ENRICHED *Leucaspius delineatus* KAIROMONE FROM ANION EXCHANGER (SAX) AT pH 5.5[a]

Treatment	Mean day depth (cm ± 95% CI)
Fish	90 ± 4
Fish	
Eluate of SAX	13 ± 4
SAX desorbed with methanol	16 ± 6
SAX desorbed with 0.5 M NaCl	88 ± 2
SAX desorbed with 1 M NaCl	17 ± 10
Control	
SAX desorbed with 0.5 M NaCl	15 ± 8

[a]Eluate: fraction that passed unretained. Desorption of the retained activity was attempted by methanol and steps of increasing salinity. C18-SPE-enriched kairomone was used for fish treatments, nonfish water was used for control treatments. Maximum mean day depth (95 cm) indicates maximum biological activity (N = 5).

acetylated primary and secondary amino groups were tested. No loss of activity was found after derivatization with fluorescamine, *o*-phthaldialdehyde, or FMOC, which showed that the loss of activity after unspecific acetylation was not due to an amino group.

Neither reduction with Pt/H_2 nor with $NaBH_4$ resulted in reduced activity (Table 4). Thus, neither a carbonyl group nor olefinic double bonds are essential for biological activity.

TABLE 4. CHEMICAL DERIVATIZATION OF KAIROMONE FROM *Leucaspius delineatus*[a]

Treatment	Mean day depth (cm ± 95% CI)	
	Fish	Control
Enriched kairomone	83 ± 3	
Saponification	86 ± 5	12 ± 7
Base-catalyzed acetylation	4 ± 4	
Acetylation and saponification	89 ± 3	15 ± 11
Acetylation with *o*-phthalaldehyde	87 ± 2	27 ± 17
Acetylation with fluorescamine	85 ± 1	13 ± 11
Acetylation with FMOC	86 ± 3	9 ± 5
Hydrogenation with Pt/H_2	86 ± 8	16 ± 19
Hydrogenation with $NaBH_4$	70 ± 4	13 ± 11

[a]C18-SPE-enriched kairomone was used for fish treatments, nonfish water was used for control treatments. Maximum mean day depth (95 cm) indicates maximum biological activity (N = 5).

TABLE 5. COMPARISON OF CHEMICAL CHARACTERISTICS OF KAIROMONES RELEASED BY THREE CYPRINID FISH SPECIES[a]

Treatment	Mean day depth (cm ± 95% CI) and kairomone released by		
	Leucaspius delineatus	*Carassius carassius*	*Rutilus rutilus*
Holding water	83 ± 13	79 ± 7	81 ± 9
C18-SPE, methanol	89 ± 4	92 ± 2	87 ± 9
Anion exchanger: eluate	13 ± 4	8 ± 10	12 ± 11
Anion exchanger: 1 M NaCl	88 ± 2	89 ± 1	92 ± 3
Acetylation	4 ± 4	6 ± 3	7 ± 4
Acetylation and saponification	89 ± 3	59 ± 5	86 ± 11

[a]Maximum mean day depth (95 cm) indicates maximum biological activity ($N = 5$).

Chemical Comparison of Kairomones from Different Cyprinid Species. When the kairomone released by *Leucaspius delineatus* was compared chemically with those of two different cyprinid species (*Carassius carrassius* and *Rutilus rutilus*), no differences were found (Table 5) with regard to retention by C18-SPE, anion exchanger, and reversible inactivation by acetylation. When an aliquot of C18-enriched kairomone from the three cyprinid species was subjected to HPLC on a linear water–methanol gradient and fractions were tested for induction of DVM, activity was confined to the same fraction in all three cases (Figure 1). The activity of the active HPLC fraction matched that of the aliquot prior to HPLC, indicating that total activity was recovered.

DISCUSSION

Many species of planktivorous fish are known to exude a chemical cue inducing DVM in *Daphnia*. First attempts to chemically characterize the kairomone released by *Leucaspius delineatus* showed that it is a water-soluble, low-molecular-weight compound (< 500 daltons), which is stable to extreme pH and temperature (Loose et al., 1993). On the basis of those results we devised a method for enriching the kairomone from the holding water of fish by solid-phase extraction (SPE). The cue was quantitatively removed from the holding water of fish by SPE, both with weakly (phenyl, C8) and with strongly lipophilic (C18) resins. Nevertheless hydrophilic groups are present in the kairomone rendering it not extractable by diethyl ether at any pH.

An anionic side group, which is not a carboxyl group, contributes to the

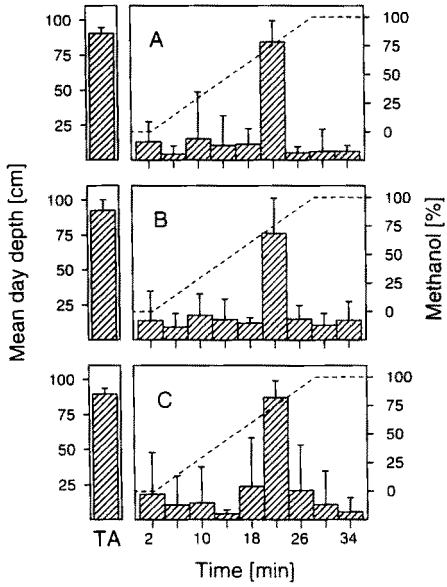

FIG. 1. Reversed-phase HPLC of enriched kairomones from different cyprinid fish species. Bars indicate biological activity (error bars: 95% CI, $N = 5$). The broken line indicates the methanol gradient. TA: total activity prior to HPLC. Fractionation of TA from A: *Leucaspius delineatus*, B: *Carassius carassius*, C: *Rutilus rutilus*. HPLC of kairomones from the three species yields one active fraction with identical retention times. Main activity of the HPLC matches total activity.

hydrophilic character of the cue. Additional nonionic hydrophilic groups are part of the kairomone, as can be concluded from the reversible inactivation by acetylation, indicating the presence of hydroxyl, amino, or thiol groups (Knapp, 1979) that are essential for activity. The presence of amino groups is ruled out since agents selectively blocking primary and secondary amino groups did not inactivate the cue. These findings are supported by the lack of interactions with a strong cation exchange resin at neutral and acidic pH, as would be expected for a protonated amino group. Hence, it is probably a hydroxyl group that is essential for activity. As a nonionic group, it also contributes under in situ conditions of pH 8–10 to the hydrophilic character of the kairomone.

The kairomone released by *Leucaspius delineatus* is of low-molecular weight and intermediate lipophilicity and can be reversibly inactivated by acetylation. Amino groups are not part of the molecule, and neither olefinic and ester bonds nor aldehyde and keto groups are essential for biological activity. The nonvolatile, anionic nature of the DVM-inducing chemical released by

L. delineatus ensures that the cue spreads fast and lingers in the epilimnion. This characteristic is adequate for signaling the activity of planktivorous fish, which usually forage throughout the whole epilimnion and are continuously active over a period of months (Ringelberg et al., 1991).

Water-soluble kairomones of low volatility have been found in other aquatic predator–prey systems as well: predatory rotifers release kairomones acting on other rotifers (Gilbert, 1966), *Chaoborus* releases a kairomone that acts on *Daphnia* (Tollrian and von Elert, 1994), and ciliates respond to kairomones from turbellarian flatworms (Kuhlmann, 1994), predatory ciliates (Kusch and Heckmann, 1992), and rhizopods (Kusch 1993).

The release of a kairomone that induces DVM in *Daphnia* occurs in many plantivorous fish of different taxa (for a review see Larsson and Dodson, 1993), indicating that DVM is not a predator-specific defense mechanism. This unspecificity of DVM leads to the question: is it the same kairomone in different species that induces the response? We chemically compared the kairomones of three different cyprinid species. No differences were found, when the kairomone of *Leucaspius delineatus* was compared with those of *Carassius carassius* and *Rutilus rutilus* by using both chemical characterization and HPLC. However, due to the steep gradient of the HPLC, it is possible that the kairomones behave chromatographically similarly, but are chemically different. However, the data presented suggest that the investigated cues are chemically similar, if not identical. As all planktivorous fish represent a risk of predation to *Daphnia*, a common rather than a species-specific cue should be preferred as an indicator of the overall risk of predation. No evidence for the involvement of more than one cue in the induction of DVM in *Daphnia* has been found.

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ANTIFUNGAL POLYOXYGENATED FATTY ACIDS FROM *Aeollanthus parvifolius*

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Abstract—Two polyoxygenated fatty acids with antifungal activity have been isolated from aerial parts of *Aeollanthus parvifolius* (Labiatae) using bioassay-guided isolation procedures. Spectroscopic analysis was used to identify the compounds as (*Z*)-4ξ,9ξ-diacetoxy-6ξ,7ξ-epoxy-5ξ-hydroxy-8-oxododeca-2-enoic acid and (*Z*)-4ξ,6ξ,7ξ,9ξ-tetraacetoxy-5ξ-hydroxy-8-oxododeca-2-enoic acid. Both of these compounds inhibited spore germination of *Cladosporium cucumerinum*. Minimum inhibitory doses were 1 μg and 5 μg, respectively, but only the former inhibited germination of *Aspergillus niger* at a dose of 5 μg. Neither compound exhibited antibacterial activity. (*Z*)-4ξ,9ξ-Diacetoxy-6ξ,7ξ-epoxy-5ξ-hydroxy-8-oxododeca-2-enoic acid reduced the hyphal growth of *Pythium ultimum*. The mode of action was not resolved but did not involve either the disruption of the cell wall membrane or the inhibition of extracellular enzymes. Application of (*Z*)-4ξ,6ξ,7ξ,9ξ-tetraacetoxy-5ξ-hydroxy-8-oxododeca-2-enoic acid to seedling plants did not give any protection against fungal attack, and indeed the compound appeared to be phytotoxic.

Key Words—*Aeollanthus parvifolius*, Labiatae, polyoxygenated fatty acids, antifungal activity, *Cladosporium cucumerinum*, *Aspergillus niger*, *Pythium ultimum*.

INTRODUCTION

The genus *Aeollanthus* Mart. ex Spreng (Labiatae) consists of over 100 species and occurs throughout Africa (Ryding, 1986). *Aeollanthus parvifolius* Benth. is

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a subshrub, sometimes tiny and almost herbaceous, which grows on rocks or shallow soil at altitudes of 0–1300 m in parts of Transvaal, Natal, Swaziland, and southern parts of Mozambique (Ryding, 1986). This species was selected for study based on results obtained from random screening of several species of the Labiatae, when the chloroform extract was found to display appreciable antifungal activity. The compounds responsible for activity were identified as novel (*Z*)-dodeca-2-enoic acid derivatives, more highly oxygenated than those found previously in another species of *Aeollanthus* (Dellar et al., 1996). Attempts were made to determine the mode of action of the two new fatty acids against some plant pathogenic fungi.

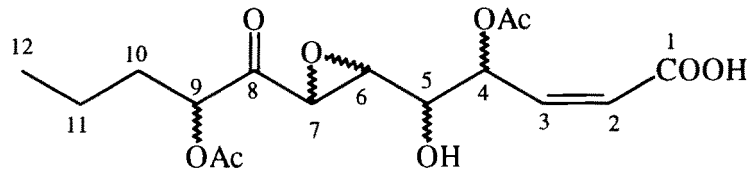
METHODS AND MATERIALS

Aeollanthus parvifolius was obtained from The Living Collection at The Royal Botanic Garden, Edinburgh, where a voucher specimen is deposited. The material used in this study was propagated in glasshouses at Strathclyde University.

Determination of Antifungal Activity. Fresh plants were lyophilized and powdered to yield 60 g of dry plant material, which was then extracted in chloroform followed by methanol at room temperature for 72 hr, yielding 5 g and 3.6 g of dry extract, respectively. Both extracts were subjected to vacuum liquid chromatography (VLC) (Pelletier et al., 1986) and the resulting fractions were investigated for antifungal activity using direct bioautography on silica gel TLC plates with *Cladosporium cucumerinum* (IMI 299104) as the test organism (Homans and Fuchs, 1970). Antifungal activity was observed in the chloroform extract in fractions eluted from the VLC column with 35–60% petroleum ether in ethyl acetate.

Isolation of Antifungal Compounds. Direct bioautography of the active fractions on silica gel using hexane–ethyl acetate (1 : 1) as the developing solvent revealed two fungitoxic bands at R_f 0.22 and 0.45. Purification was achieved with centrifugal thin-layer chromatography (Marston and Hostettmann, 1991) using an isocratic elution system of hexane–ethyl acetate (3 : 2). Compounds **1** and **2** (Figure 1) were isolated in yields of 43.5 mg and 400 mg, corresponding to R_f values of 0.45 and 0.22, respectively.

Identification of Isolated Compounds. (*Z*)-4ξ,9ξ-Diacetoxy-6ξ,7ξ-epoxy-5ξ-hydroxy-8-oxododeca-2-enoic acid (**1**) is an oil, giving a red-brown spot when sprayed with 1% vanillin in concentrated sulfuric acid and heated to 100°C for 5–10 min. $[\alpha]_D$ = +7.1° (*c* 0.14, MeOH). IR γ_{max} (KBr) cm^{-1}: 3422, 2964, 1741, 1374, 1221, 1198, 1096, 1044, 953, 826. UV λ_{max} (MeOH) nm: 272, 202. For NMR, see Table 1. HR-FAB-MS *m/z*: 363 $(M\text{-}H_2O+Na)^+$. (*Z*)-4ξ,6ξ,7ξ,9ξ-Tetraacetoxy-5ξ-hydroxy-8-oxododeca-2-enoic acid (**2**) is an



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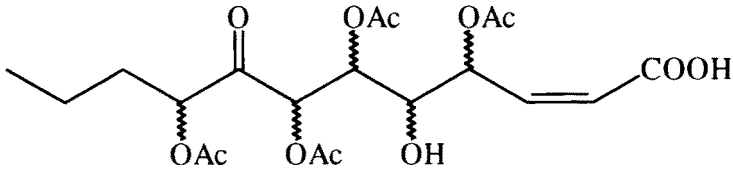

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FIG. 1. Compounds **1** and **2**.

oil, giving a red–brown spot when sprayed with 1% vanillin in concentrated sulfuric acid and heated to 100°C for 5–10 min. $[\alpha]_D$ +147.8° (*c* 1.84, MeOH). IR γ_{max} (KBr) cm^{-1}: 3442, 2962, 1751, 1374, 1222, 1094, 1033, 949, 826, 757. UV λ_{max} (MeOH) nm: 332, 272, 212. For NMR, see Table 1. HR-FAB-MS *m/z*: 465 $(M\text{-}H_2O+Na)^+$.

Determination of Minimum Inhibitory Dose (MID) and Minimum Inhibitory Concentration (MIC). The MID required to prevent fungal spore germination was determined using direct bioautography with *C. cucumerinum* and *Aspergillus niger* (IMI 17454) as test organisms. This was undertaken as previously described for *C. cucumerinum* (Homans and Fuchs, 1970), while for *A. niger* a sporing colony was resuspended in sterile saline (20 ml) and a 100-μl aliquot was added to 10 ml of molten malt extract agar. This *A. niger*-seeded medium was sprayed thinly and evenly over a silica gel plate previously impregnated with the test compounds at a range of concentrations, together with amphoteracin-B as a control. The plates were incubated at 25°C for 48 hr. The lowest dose at which no sporulation was observed was determined as the MID.

The antibacterial MIC value was assessed using the broth dilution method (Rios et al., 1988), with *Bacillus subtilis* (NCTC 8326) (4×10^{13} CFU/ml), *Staphylococcus aureus* (NCTC) 6751 (9×10^{13} CFU/ml), *Pseudomonas aeruginosa* (NCTC 6750) (9×10^{13} CFU/ml), *Erwinia carotovora* (IMI 347390) (4×10^9 CFU/ml), *Pseudomonas syringae* (IMI 349170) (4×10^9 CFU/ml),

TABLE 1. ^{13}C AND ^{1}H NMR ASSIGNMENTS FOR **1** AND **2**[a]

	^{13}C		^{1}H	
C/H	1	2	1	2
1	160.8	160.8		
2	125.0	125.4	6.15 d (9.8)	6.20 d (9.7)
3	140.3	140.0	7.05 dd (9.7, 5.8)	6.98 dd (9.6, 6.2)
4	62.5	59.7	5.27 dd (5.8, 2.7)	5.26 dd (6.1, 2.5)
5	73.9	75.0	4.26 dd (7.9, 2.6)	4.71 dd (9.7, 2.5)
6	54.9	66.1	3.65 dd (8.0, 4.4)	5.82 dd (9.7, 1.6)
7	55.2	72.9	3.94 d (4.4)	5.58 d (1.6)
8	200.9	199.7		
9	77.4	76.2	5.16 dd (9.0, 4.2)	5.44 dd (8.9, 3.8)
10	31.9	32.7	1.72-1.89 m	1.76/1.9 m
11	18.7	18.5	1.43 m	1.4 m
12	13.6	13.7	0.95 t (7.4)	0.93 t (7.3)
4-OCO Me	20.6	20.38-20.64	2.13 s	2.14 s
4-OCOMe	169.7	170.0		
6-OCOMe		20.38-20.64		2.05 s
6-OCOMe		169.4		
7-OCOMe		20.38-20.64		2.13 s
7-OCOMe		169.6		
9-OCOMe	20.6	20.38-20.64	2.09 s	2.05 s
9-OCOMe	170.8	170.0		

[a]Coupling constants (J in hertz). Spectra run in deuteriochloroform on a Bruker AMX400 instrument.. s = singlet, d = doublet, dd = double doublet, t-triplet, m = multiplet

and *Streptomyces scabies* (IMI 349428) (3×10^8 CFU/ml) as the test organisms. The former three species were incubated at 37°C and the remainder at 25°C. Test compounds were dissolved in methanol and added in 10-μl aliquots to each well in a 96-well microtiter plate giving a final concentration range of 0.01–100 μg/ml. Chloramphenicol in the same concentration range was used as the positive control and 10 μl methanol alone as negative control. Results were recorded after 24 hr.

Investigation of Reduction of Radial Hyphal Growth in Response to Test Compounds. Test compounds were added in 1-μl aliquots of methanol to 10-ml batches of molten malt extract agar to give final concentrations of 5, 10, 25, 50, and 100 μg/ml. Methanol-only and agar-only negative controls were performed in parallel, as were positive controls using griseofulvin, at a dose of 10 μg/ml. The agar was allowed to solidify in 9-cm Petri dishes, and each plate was inoculated at the center with 6-mm fungal plugs taken from the margins of actively growing colonies of either *Pythium ultimum* (IMI 30827) or *Thaneto-*

phorus cucumeris (IMI 303152). Plates were incubated for 48–72 hr at 25°C and radial growth for each test was recorded by taking the average diameter from four measurements.

Investigation of Effect of Compounds on Hyphal Morphology. Test compounds were dissolved in methanol and added in 1-μl aliquots to 1-ml portions of molten malt extract agar, to given final concentrations of 10, 25, 50, and 100 μg/ml. Each batch was then applied to a sterilized glass slide as an even layer and placed in a Petri dish with damp filter paper (7 cm ID). A 6-mm plug taken from cultures of *P. ultimum* or *T. cucumeris* was placed onto the center of each slide. Dishes were covered and incubated at 25°C for 18–48 hr. Hyphae were examined with light microscopy for changes in hyphal morphology, such as abnormal growth patterns or lysis of hyphal tips and were compared with methanol-only and agar-only negative controls. Staining of cells with Evan's blue stain (0.05% in distilled water) was used to indicate cell membrane dysfunction (Smith, 1982).

Determination of Effect of Test Compounds on Extracellular Enzymes. The method described by Barth and Bridge (1989) was used to detect whether the antifungal activity of test compounds was due to the inhibition of β-glucosidase, which is released as an extracellular enzyme into the culture media. The substrate used was 4-methylumbelliferyl-β-D-glucoside (4MUGlu) (Sigma) prepared according to Barth and Bridge (1989). Malt extract broth (100 ml) was added to conical flasks (250 ml) and autoclaved. Test compounds dissolved in 100-μl aliquots of methanol were added to give a final concentration of 50 μg/ml. Negative controls consisted of broth and methanol alone and broth alone. Each flask was subsequently inoculated with 2 × 6-mm disks taken from the leading edge of an actively growing vegetative culture of *P. ultimum*. Flasks without the addition of fungi were run as further controls. Each flask was sealed with a plug of cotton wool and placed in a shaker–incubator at 25°C (100 rpm) for five days. Cultures were vacuum filtered and allowed to air dry for 24 hr, after which each fungal mass was recorded. Culture filtrates were assayed for enzyme activity as follows. Substrate (50 μl) was added to culture medium (50 μl) in a microtiter well. Each test was performed in triplicate. Plates were then incubated at 37°C for 4 hr. To examine whether reduction of enzyme activity was due to direct enzyme inhibition by the test compounds, culture filtrate taken from the fungus-only flask was assayed as above, but with the direct addition of the test compound at final concentrations of 25, 50, and 100 μg/ml.

After incubation, 50 μl of saturated sodium bicarbonate solution was added to each well in order to enhance fluorescence of any 4-methyl umbelliferone (4MU) released (O'Brien and Colwell, 1987). Fluorescence was measured at excitation and emission wavelengths of 377 and 447 nm, respectively. Readings were compared with a standard curve prepared using recrystallized 4MU dissolved in methanol over a concentration range of 0.01–10 μM.

Investigation of In Vivo Activity of Test Compounds. Compounds were investigated for in vivo activity using a plant/pathogen test system of cucumber seedlings (Mr. Fotheringills Ridge Perfection) and *Cladosporium cucumerinum*. Test solutions were prepared in acetone–water (1 : 1) to give a final concentration of 100 μg/ml and seedlings at the two-cotyledon stage were sprayed until runoff. Control plants were sprayed with acetone–water (1 : 1) solution only, 1% malt extract broth only, or remained untreated. After allowing leaves to dry, half the seedlings subjected to each test were sprayed until runoff with a spore suspension of *C. cucumerinum* in 1% malt extract broth (2.5×10^6 spores/ml) (Emmatty, 1974). All seedlings were covered with polythene for 48 hr at approximately 18°C, after which the plastic was removed and plants were grown under normal glasshouse conditions. The subsequent onset of disease was monitored.

RESULTS

Identification of Compounds 1 and 2. Compounds **1** and **2** showed similar spectral features, which were analogous to those observed previously for polyoxygenated fatty acids isolated from another species of *Aeollanthus* (Dellar et al., 1996). The FT-IR spectra of both indicated carbonyl and hydroxyl functional groups. The ^1H and ^{13}C NMR spectra of **1** (Table 1) revealed the presence of a dodecanoic acid, with one double bond and two acetoxyl substituents. A FAB-MS spectrum obtained for **1** failed to show a molecular ion, but a major fragment at *m/z* 363 was identified as due to $(M\text{-}H_2O+Na)^+$, indicating a molecular weight of 358 ($C_{16}H_{22}O_9$). Based on the coupling constant of the olefinic protons (J = 9.8 Hz), the double bond was determined to have *cis* stereochemistry. A comprehensive study using two-dimensional (2-D) NMR techniques was undertaken applying methods described previously by Quader et al. (1991). Direct H-C couplings were established on the basis of HC-COBI-dec experiments, while the 2-D ^1H-^1H COSY-45 spectrum allowed the assignment of ^2J and ^3J homonuclear correlations, and long-range hetronuclear couplings were determined by the HMBC technique (Table 2). These observations established the position of the double bond at C-2/C-3, placed the acetoxyl substituents at C-4 and C-9, and the carbonyl at C-8. The presence of a terminal propyl group (C-10 to C-12) required that C-5 to C-7 be oxygenated. The C-5 carbon had to be assigned the ^{13}C resonance at 73.9 ppm because of long-range coupling to the olefinic H-3 (Table 2) leaving C-6 and C-7 represented by the signals at 54.9 and 55.2 ppm. Of the remaining two oxygens to be assigned to C-5 through C-7, C-5 must carry a hydroxyl while the more shielded C-6 and C-7 carbons must share an epoxide. Thus compound **1** can be assigned the structure (*Z*)-4ξ,9ξ-diacetoxy-6ξ,7ξ-epoxy-5ξ-hydroxy-8-oxododeca-2-enoic acid.

Compound **2** was clearly similar to **1** but showed two additional acetoxyl

TABLE 2 ^2J AND ^3J HMBC HETERONUCLEAR CORRELATIONS IN **1** AND **2**

H	**1**		**2**	
	^2J	^3J	^2J	^3J
H-2	160.8 (C1)	62.5 (C4)	160.8 (C1)	59.7 (C4)
H-3	62.5 (C4)	73.9 (C5), 160.8 (C1)	59.7 (C4)	75.0 (C5), 160.8 (C1)
H-4	140.3 (C3)	125.0 (C2), 169.7 (Ac)	140.0 (C3)	125.4 (C2), 170.0 (Ac)
H-5	54.9 (C6), 62.5 (C4)	55.2 (C7)	59.7 (C4), 66.1 (C6)	
H-6	55.2 (C7), 73.9 (C5)		72.9 (C7), 75.0 (C5)	59.7 (C4), 169.4 (Ac)
H-7	54.9 (C6), 200.9 (C8)		66.1 (C6), 199.7 (C8)	75.0 (C5), 169.6 (Ac)
H-9	31.9 (C10), 200.9 (C8)	18.7 (C11), 170.8 (Ac)	32.7 (C10), 199.7 (C8)	18.5 (C11), 170.0 (Ac)
H_2-10	18.7 (C11), 77.4 (C9)	13.6 (C12), 200.9 (C8)	18.5 (C11), 76.2 (C9)	13.7 (C12)
H_2-11	13.6 (C12), 31.9 (C10)		13.7 (C12)	
H_3-12	18.7 (C11)	31.9 (C10)	18.5 (C11)	32.7 (C10)
4-OCOMe		169.7		170.0
6-OCOMe				169.4
7-OCOMe				169.6
9-OCOMe		170.8		170.0

substituents, which, using the same NMR techniques (Tables 1 and 2), must be placed at positions C-6 and C-7, therefore replacing the epoxide moiety present in **1**. The *cis* configuration (J = 9.7 Hz) at C-2 and C-3 was maintained. The FAB-MS spectrum obtained for **2** failed to show the parent ion corresponding to $C_{20}H_{28}O_{12}$ (MW 460), but the m/z 465 $(M\text{-}H_2O+Na)^+$ was present. Compound **2** was therefore identified as (Z)-4ξ,6ξ,7ξ,9ξtetraacetoxy-5ξ-hydroxy-8-oxododeca-2-enoic acid.

Minimum Inhibitory Dose of 1 and 2. The two compounds were able to prevent spore germination of *Cladosporium cucumerinum* at 1 μg and 5 μg, respectively, with activity persisting for 40 hr. Only compound **1**, however, was able to inhibit germination of *Aspergillus niger* at a dose of 5 μg, with activity again persisting for 40 hr (Table 3).

Antibacterial Activity of 1 and 2. Neither compound was able to inhibit growth of any of the bacterial species selected for this assay. They were tested to a maximum concentration of 100 μg/ml.

Inhibition of Fungal Radial Growth by 1 and 2. Regression analysis allowed ID_{50} values (the concentrations at which fungal growth was inhibited by 50% in comparison to the methanol control) to be determined after 24 hr. Compound **1** gave ID_{50} values of 24.9 μg/ml and 179.2 μg/ml against *P. ultimum* and *T. cucumeris*, respectively, while **2** was less effective, with ID_{50} values of 232.2 μg/ml and 728.2 μg/ml, respectively.

Effect of 1 and 2 on Fungal Morphology. After 22 hr, **1** caused hyphal shrinkage and curling of *P. ultimum* at concentrations of 10–25 μg/ml in comparison with negative controls, while concentrations of 50–100 μg/ml inhibited vegetative growth almost completely. However, after 44 hr, inhibition was to some degree overcome at all concentrations. Normal growth of *T. cucumeris*

TABLE 3. MINIMUM INHIBITORY DOSE OF COMPOUNDS **1** AND **2** AGAINST *Cladosporium cucumerinum* AND *Aspergillus niger*

	Minimum inhibitory dose (μg)		
Time (hr)	**1**	**2**	Amphoteracin B
C. cucumerinum			
40	1	5	<0.5
88	1	25	<0.5
264	5	>100	<0.5
A. niger			
40	5	>100	<0.5
80	10	>100	<0.5
264	10	>100	<0.5

was also disrupted at 50–100 μg/ml after 22 hr, but no inhibition was observed after 44 hr. Compound **2** caused little growth disruption of *T. cucumeris*, but *P. ultimum* did show some signs of abnormal growth up to 22 hr, after which no inhibition was observed. Abnormal hyphae failed to take up Evan's blue stain, indicating that membranes were able to function normally.

Inhibition of Fungal Extracellular Enzymes by Compound 1. Regression analysis of fluorescence vs. 4MU concentration gave the equation $y = 1.795x + 29.751$ ($r^2 = 0.99$), which was used to calculate the release of 4MU in the enzyme assay. Compound **1** reduced release of β-D-glucosidase by 92%, while fungal biomass was reduced by 74% at a concentration of 50 μg/ml. When the cell-free culture medium obtained by filtration of fungi was incubated with compound **1** no inhibition of enzyme activity was observed. Therefore, the reduction of enzyme activity does not appear to be due to the direct action of **1.**

In Vivo Activity of Compound 1. Uninoculated seedlings treated with compound **1** remained healthy, as did plantlets treated with broth, or water and acetone alone, with and without subsequent inoculation. Only those plantlets treated with **1** prior to inoculation developed signs of disease, with 20% dying within nine days and 55% within 15 days.

DISCUSSION

Both compounds **1** and **2** are previously unreported. Results obtained from all the in vitro assays against various fungal organisms indicate compound **1** to be the more fungitoxic. In the agar dilution assay, fungal hyphal growth was able to attain the maximum allowed on a 9-cm Petri dish; therefore, the effects of **1** and **2** would appear to be fungistatic rather than fungicidal. Biological activity has been reported for compounds with similar structures from a number of other sources (Bowers et al., 1986; Masui et al., 1989: Kuwahara et al., 1992; Namai et al., 1993). A study of structure–activity relationships of various fatty acids against *Pyricularia oryzae* (Mori et al., 1987) revealed that a free monocarboxyl, a chain length of C-10 to C-13 and an α,β-unsaturated carboxylic acid moiety was necessary for any activity. Both **1** and **2** satisfy these criteria. The addition of an epoxide group in **1** seems likely to be the important factor for its enhanced activity.

Intuitively, it would seem probable that the manufacture of these compounds forms a useful chemical defense against fungal pathogens in *A. parvifolius*. However, an attempt to use pure compound **1** to protect cucumber seedlings against the attack of fungal pathogens was not successful; in fact, pretreatment of the seedlings with this compound appeared to enhance the infection process, resulting in a higher mortality rate for treated seedlings than for the controls. Similar compounds have been reported to have cytotoxic activity

(Kuwahara et al., 1992; Namai et al., 1993), which may account for the reduced success of treated seedlings. *Aeollanthus* species probably sequester these potentially toxic compounds in trichomes present on the leaf surface, thereby isolating them from general metabolism, but at the same time leaving them available for rapid release in order to fight pathogen invasion.

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REPELLENCY OF ORAL EXUDATE TO EASTERN AND WESTERN SPRUCE BUDWORM LARVAE (LEPIDOPTERA: TORTRICIDAE)

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Abstract—A two-choice feeding bioassay was used to investigate the intraspecific repellency of the larval oral exudate of eastern and western spruce budworms, *Choristoneura fumiferana* (Clem.) and *C. occidentalis* Free., respectively. Results of the bioassay indicated that feeding behavior on artificial diet-drop feeding stations was modified in the presence of exudate, with feeding stations treated with conspecific exudate being avoided when an untreated station was available 3 cm away. Feeding was suppressed when a single, exudate-treated station was provided, or when the treated and untreated stations were separated by only 1 cm. The repellent effect functioned both inter- and intraspecifically. When induced to produce exudate, *C. occidentalis* larvae were not immediately repelled by either their own or other individuals' exudate. However, 24 hr after induction, test larvae were repelled by exudate from either source. In both species, larval oral exudate probably functions to repel conspecific competitors.

Key Words—*Choristoneura fumiferana*, *Choristoneura occidentalis*, spruce budworm, oral exudate, regurgitant.

INTRODUCTION

Many insects release oral secretions when they are disturbed or handled (Davies and McCauley, 1970; Corbet, 1971; Eisner et al., 1974). Such secretions are an important defense against predators and parasites (Eisner et al., 1974). However, they may also play a role in intraspecific interactions, mediating optimal

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larval density in a habitat in a concentration-dependent manner (Corbet, 1971). Recent evidence (Poirier and Borden, 1995) has shown that oral exudate from larvae of eastern and western spruce budworms, *Choristoneura fumiferana* (Clem.) and *Choristoneura occidentalis* Free., respectively, may act as an epideictic (spacing) pheromone (Prokopy, 1981). Larvae produced the exudate during intraspecific encounters, and experimental exposure to exudate caused an increase in the proportion of larvae dispersing from established feeding sites (Poirier and Borden, 1995). In the field, generally only one larva is found in each tree bud (L. E. Maclauchlan, British Columbia Ministry of Forests, Kamloops Region, personal communication).

In order for the intraspecific repellency of larval oral exudate to be investigated further, an effective laboratory bioassay procedure was required. Our first objective, therefore, was to develop such a bioassay. Subsequent objectives were to determine the specificity of the exudate between the two *Choristoneura* species and to determine whether an individual larva could "recognize" whether exudate came from itself or another larva.

METHODS AND MATERIALS

Insects. Second-instar, diapausing larvae of *C. fumiferana* and *C. occidentalis* were obtained from the Forest Pest Management Institute, Canadian Forest Service, Sault Ste. Marie, Ontario. These insects were reared in the laboratory at approximately 24°C and 60% relative humidity. Photoperiod was maintained at 16L:8D. Larvae were reared on an agar-based, artificial spruce budworm diet (Bio-Serv Inc., Frenchtown, New Jersey) in 30-ml disposable vials. Two larvae were kept in each vial. Some *C. occidentalis* larvae were reared on cut branches of Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco, collected in Burnaby, British Columbia.

Bioassay Development. To test the repellent effect of larval oral exudate, two drops of molten artifical diet (approximately 0.5 cm in diameter) were placed in the bottom of a 100 × 15-mm disposable Petri dish and allowed to solidify. In all bioassays, the Petri dishes were inverted so that the diet feeding stations were on the "ceiling" of the Petri dish chamber.

In experiments 1 and 2, the two feeding stations were placed in each dish with 3 cm between their centers. In experiments 3 and 4, the two feeding stations were separated by only 1 cm. Each dish for experiments 5 and 6 contained a single feeding station, placed at the center of the chamber. Oral exudate was collected from artificial diet-reared larvae by touching their heads with a 5-μl micropipet (Poirier and Borden, 1995). As much exudate as could be collected from two larvae was used to cover one of the two feeding stations in each dish

in experiments 1–4, and the single stations in experiments 5 and 6. Separate dishes, with one station treated with 5 μl of distilled water, served as controls.

A conspecific artificial-diet-reared third to fifth instar, (*C. fumiferana* in experiments 1, 3, and 5, and *C. occidentalis* in experiments 2, 4, and 6) was then placed on moist filter paper on the floor of each chamber and exposed to the feeding stations by placing the roof on the chamber. Test larvae that produced exudate while being transferred to the chamber were not used in the experiments. The larvae were left for 24 hr under the above rearing conditions, and their final positions were then recorded. Any evidence of feeding or establishment on a particular station, such as feeding cavities, deposited silk, silk feeding tunnels, or frass, was recorded. Larvae that did not establish on a feeding station were included in the sample, unless they had molted or pupated over the 24-hr test period. Larvae were, therefore, categorized as not feeding, feeding on the untreated station, or feeding on the treated station.

Each experiment consisted of 20 experimental and 20 control replicates. The numbers of larvae in the three categories were compared between experimental and control dishes using Fisher's exact test, $\alpha = 0.05$, for a 2×3 contingency table, due to the small sample size (Steel and Torrie, 1980; Schlotzhauer and Littell, 1987).

Interspecific Repellency. Artificial-diet-reared third to fifth instars of *C. fumiferana* (experiment 7) and *C. occidentalis* (experiment 8) were tested in Petri dish bioassays as described in experiment 1. Exudate from two larvae of one species was used to treat one of the stations in each dish. Larvae of the other species were then used as test insects. Control dishes were also set up, with one station treated with 5 μl of distilled water. There were 20 experimental and 20 control dishes for each species. Test larvae were left in the dishes for 24 hr, and the results were assessed and analyzed as in experiment 1.

Self-Recognition. Bioassay dishes for experiment 9 were constructed as dscribed in experiment 1, except that the filter paper was omitted and the dishes were not inverted. Both exudate donors and test insects were third to fifth instars of *C. occidentalis* that had been reared on artificial diet. Dishes were divided among four treatments. In the control treatment, one station in each dish was treated with 2 μl of distilled water, and the test larva was not induced to produce exudate prior to the start of the experiment. In the first experimental treatment, one station was treated with 2 μl of conspecific exudate, and the test larva was not induced. Test larvae were induced to produce exudate in experimental treatments 2 and 3; one station in each dish received 2 μl of exudate, which was produced by either the test larva (treatment 2) or another, conspecific larva (treatment 3). Test larvae were placed in all dishes immediately following treatment of the feeding stations. Each of the four treatments was replicated 40 times.

This experiment was repeated (experiment 10) with dishes that had been treated and left on a bench for 24 hr before test larvae were introduced. Test larvae were selected and, where appropriate, induced at the time of treatment and placed in individual vials of artificial diet for 24 hr, then placed in the bioassay dishes. Each of the four treatments was replicated 40 times. All test larvae from experiments 5 and 6 were left in the bioassay dishes for 24 hr. The results were then assessed and analyzed as for experiment 1.

Experiments 9 and 10 were repeated (experiments 11 and 12) using third- to fifth-instar *C. occidentalis* larvae that had been reared on foliage. Test larvae in experiment 12 were placed on small twigs of current-year Douglas fir foliage and held in individual vials for 24 hr before being placed in the bioassay dishes. For both experiments 11 and 12, each treatment was replicated 40 times. Test larvae were left in the bioassay dishes for 24 hr, and the results were then assessed and analyzed as for experiment 1.

RESULTS

Bioassay Development. When the two feeding stations were separated by 3 cm (experiments 1 and 2), the numbers of larvae in the three categories (not feeding, feeding on the untreated station, or feeding on the treated station) were significantly different between experimental and control dishes for both *C. fumiferana* and *C. occidentalis* (Figure 1). In experimental dishes, most larvae fed on the untreated stations, while in the control dishes there were approximately equal numbers feeding on the water-treated stations and the untreated stations.

When the two feeding stations were separated by 1 cm in experiments 3 and 4, the numbers of larvae in the three categories were again significantly different between the experimental and control dishes for both species (Figure 2). As in experiment 1, very few larvae fed on the exudate-treated station. However, 50% and 75% of *C. fumiferana* and *C. occidentalis*, respectively, did not feed at all on either station in the experimental dishes. Feeding on untreated and water-treated stations in control dishes was relatively equal.

When only one feeding station treated with conspecific oral exudate was available (experiments 5 and 6), 65% and 90% of *C. fumiferana* and *C. occidentalis*, respectively, did not feed (Figure 3). Approximately 90% of all larvae of both species fed on the water-treated station in control dishes (Figure 3).

Interspecific Repellency. The results of experiments 7 and 8 (Figure 4) were very similar to those of experiments 1 and 2 (Figure 1), indicating reciprocal activity of larval oral exudate between the two *Choristoneura* species. The effect of *C. fumiferana* exudate on *C. occidentalis* larvae was particularly strong; no *C. occidentalis* larvae fed on diet stations treated with *C. fumiferana* exudate, and 45% did not feed at all.

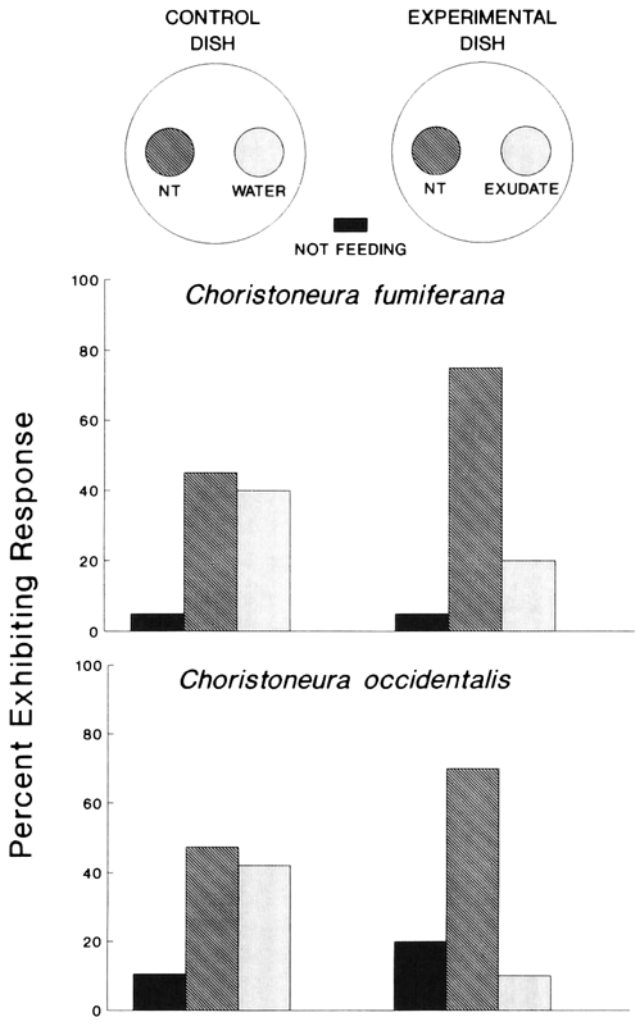

FIG. 1. Percentage of *C. fumiferana* (experiment 1) or *C. occidentalis* (experiment 2) larvae not feeding or feeding on one of two diet stations with centers separated by 3 cm. Circular schematic diagrams indicate placement of diet stations (magnified in size) in Petri dishes, with shading of treatment corresponding to the percent response bars below. The control dish (left) contains an untreated diet station (NT) paired with one treated with distilled water. The experimental dish (right) contains an untreated diet station (NT) paired with one treated with conspecific larval oral exudate. $N = 20$. For both species, control and experimental distributions are significantly different, Fisher's exact test, $P = 0.034$ and $P = 0.029$, respectively.

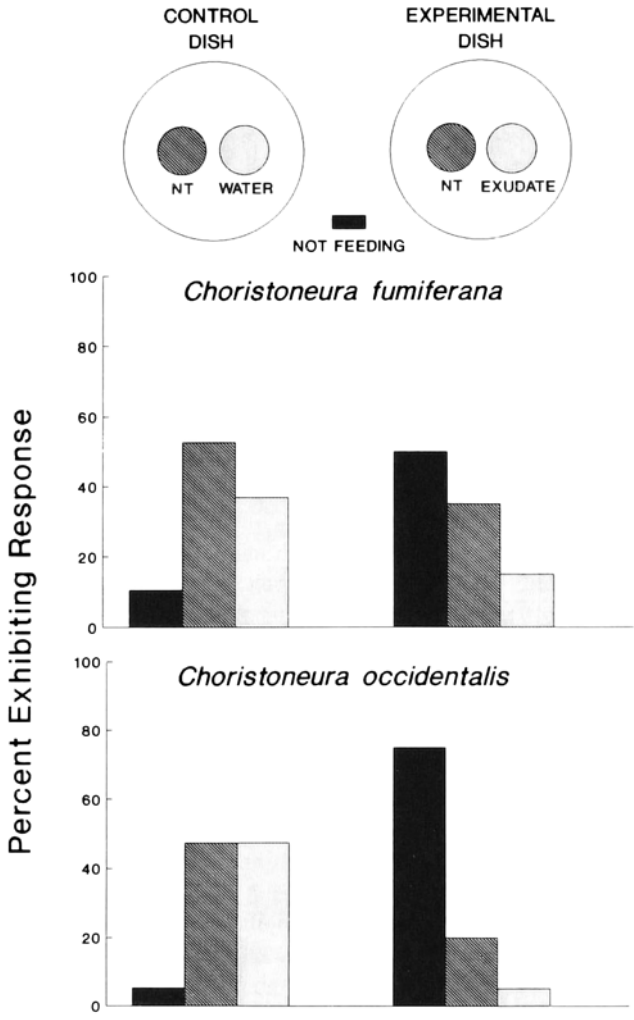

FIG. 2. Percentage of *C. fumiferana* (experiment 3) or *C. occidentalis* (Experiment 4) larvae not feeding or feeding on one of two diet stations with centers separated by 1 cm. Circular schematic diagrams indicate placement of diet stations (magnified in size) in Petri dishes, with shading of treatment corresponding to the percent response bars below. The control dish (left) contains an untreated diet station (NT) paired with one treated with distilled water. The experimental dish (right) contains an untreated diet station (NT) paired with one treated with conspecific larval oral exudate. $N = 20$. For both species, control and experimental distributions are significantly different, Fisher's Exact Test, $P = 0.026$ and $P < 0.0001$, respectively.

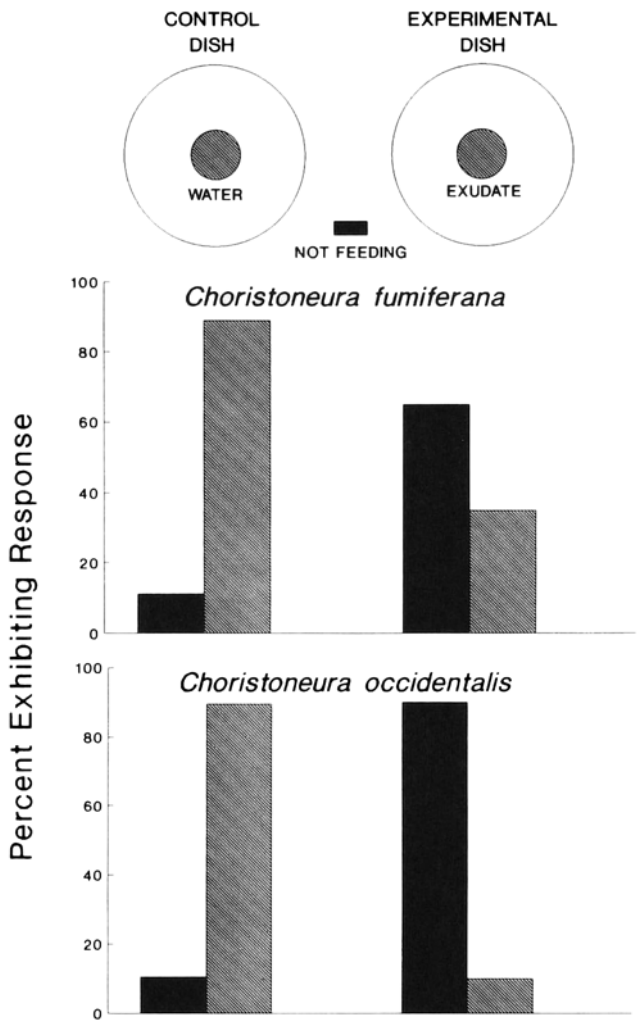

FIG. 3. Percentage of *C. fumiferana* (experiment 5) or *C. occidentalis* (experiment 6) larvae not feeding or feeding on single diet stations. Circular schematic diagrams indicate placement of diet stations (magnified in size) in Petri dishes, with shading of treatment corresponding to the percent response bars below. The control dish (left) contains diet station treated with distilled water. The experimental dish (right) contains diet station treated with conspecific larval oral exudate. $N = 20$. For both species, control and experimental distributions are significantly different, Fisher's exact test, $P < 0.001$ and $P < 0.0001$, respectively.

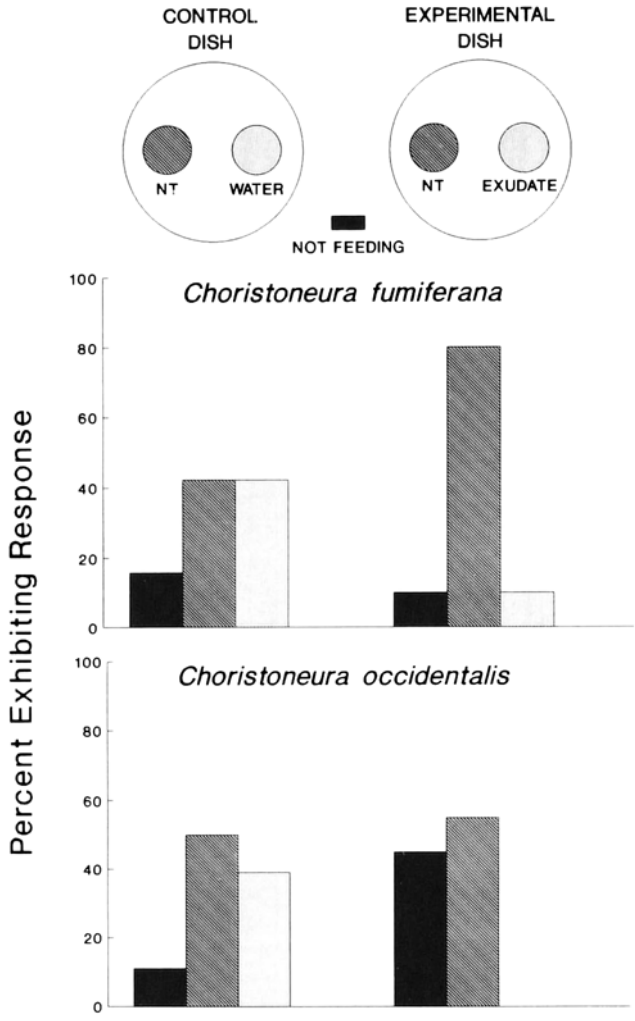

FIG. 4. Percentage of *C. fumiferana* (experiment 7) or *C. occidentalis* (experiment 8) larvae not feeding or feeding on one of two diet stations with centers separated by 3 cm. Circular schematic diagrams indicate placement of diet stations (magnified in size) in Petri dishes, with shading of treatment corresponding to the percent response bars below. The control dish (left) contains an untreated diet station (NT) paired with one treated with distilled water. The experimental dish (right) contains an untreated diet station (NT) paired with one treated with larval oral exudate from the other species. $N = 20$. For both species, control and experimental distributions are significantly different, Fisher's exact test, $P = 0.042$ and $P < 0.003$, respectively.

Self-Recognition. In experiment 9 (Figure 5), the numbers of artificial-diet-reared larvae in the three categories were significantly different from the control dishes in only one case: when one station was treated with oral exudate from another larva, and the test larva had not been induced to produce exudate, most test larvae fed on the untreated station. There was no significant difference between the treatment groups that used induced test larvae and the control groups, whether the exudate was from the test larva (Figure 5, experimental dish 2) or another larva (experimental dish 3). However, in experiment 10, when larvae were placed in the dishes 24 hr after the stations were treated, all experimental treatments caused significant rejection of the treated feeding stations. Both induced and uninduced larvae fed on the untreated station most frequently, whether the exudate was from the test larva or not.

Results with foliage-reared larvae in experiment 11 and 12 (Figure 6) were similar to those for diet-reared larvae (Figure 5).

DISCUSSION

The significant inhibition of feeding on diet stations treated with conspecific oral exudate when experimental and control stations were separated by 3 cm (Figure 1), indicates that the oral exudate acts as a feeding deterrent. The inhibition of feeding on both treated and untreated stations when the stations were 1 cm apart (Figure 2), suggests that the larval oral exudate also acts as a volatile repellent. The power of the repellency and feeding deterrency is demonstrated by the high numbers of non-feeding larvae when they were presented with no choice other than a treated diet station (Figure 3). However, these experiments used relatively large quantities of exudate, approximately 4–5 larval equivalents per treated station (Poirier and Borden, 1995), and the response may be correspondingly greater than might be expected under normal field conditions. Moreover, larvae were kept in a small chamber throughout the experiment and were not given the opportunity to disperse away from the exudate. Dispersal has already been shown to be a common larval response to an encounter with exudate (Poirier and Borden, 1995).

Both *C. fumiferana* and *C. occidentalis* larvae were repelled by exudate from larvae of the other species. Thus, the components in the exudate may be the same for both species. However, particularly if the exudate is a regurgitant (Poirier and Borden, 1995), this reciprocal activity may not be evident under field conditions, in which larvae of the two species are found on different host trees and are allopatric (Harvey, 1985).

Immediately after being induced to produce oral exudate, larvae apparently enter a refractory phase, during which they are unable to respond to their own or other individuals' exudate (Figure 5). After 24 hr, however, induced larvae had recovered their ability to discriminate. The lack of response may be due to

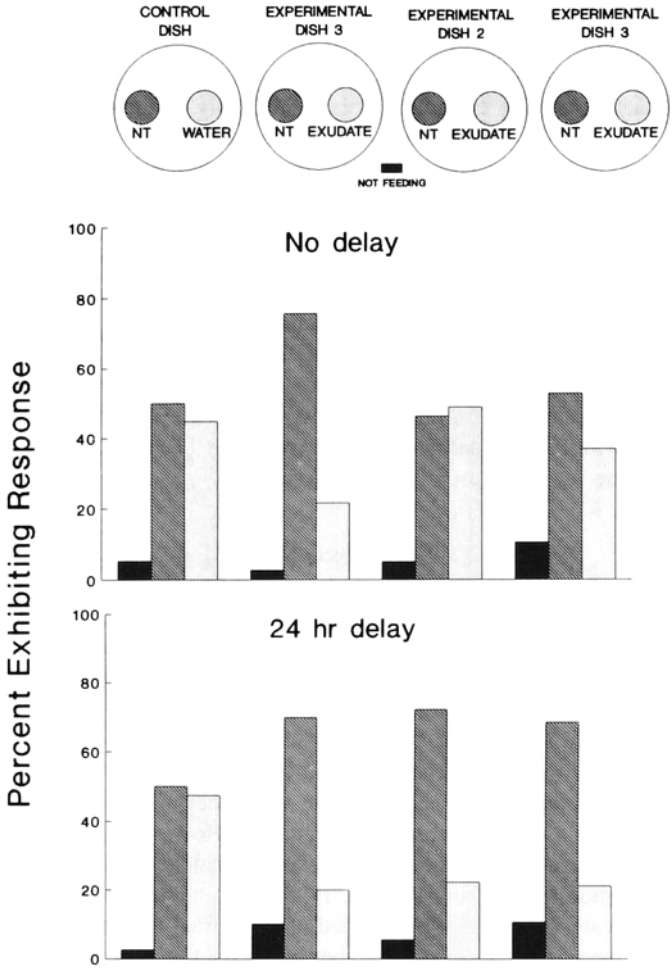

FIG. 5. Percentage of artificial diet-reared *C. occidentalis* larvae not feeding or feeding on one of two diet stations with centers separated by 3 cm, either 0 (experiment 9) or 24 hr (experiment 10) after treatment of stations and induction of exudate production. Circular schematic diagrams indicate placement of diet stations (magnified in size) in Petri dishes, with shading of treatment corresponding to the percent response bars below. The control dish (left) contains an untreated diet station (NT) paired with one treated with distilled water. The experimental dishes (right) each contain an untreated diet station (NT) paired with one treated with conspecific larval oral exudate. In dish 1, exudate was from another individual (test larva not induced to produce exudate). In dish 2, exudate was from the test larva (test larva induced). In dish 3, exudate was from another individual (test larva induced). $N = 40$. With no delay in introduction of test larva, only the distribution in dish 1 is significantly different from the control, Fisher's exact test, $P = 0.047$. Following a 24-hr delay, all experimental distributions are significantly different from the control, $P = 0.024$, $P = 0.048$, and $P = 0.035$, respectively.

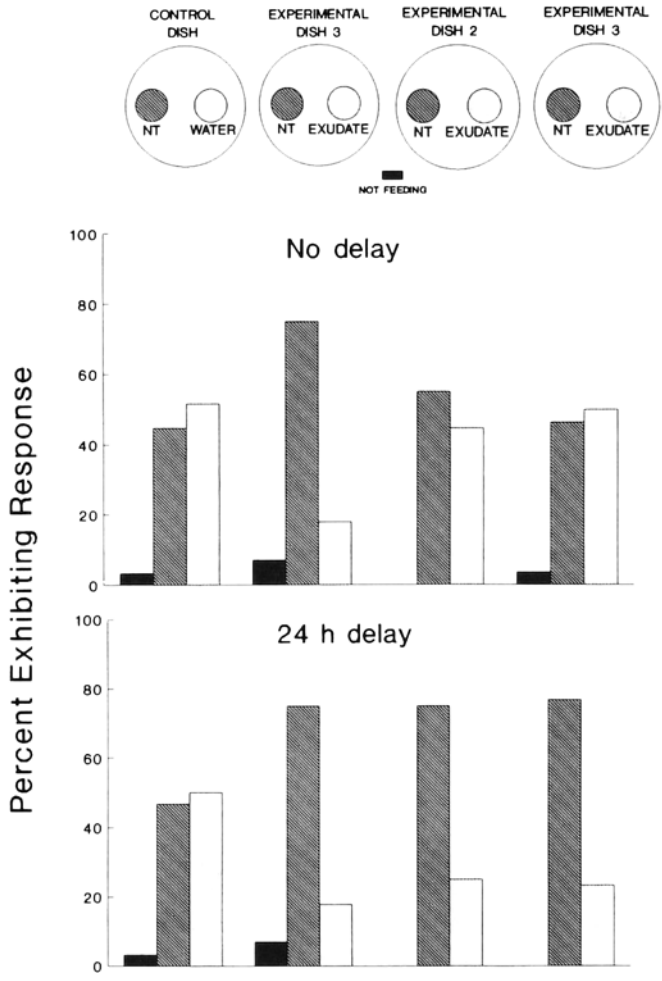

FIG. 6. Percentage of foliage-reared *C. occidentalis* larvae not feeding or feeding on one of two diet stations with centers separated by 3 cm, either 0 (experiment 11) or 24 hr (experiment 12) after treatment of stations and induction of exudate production. Circular schematic diagrams indicate placement of diet stations (magnified in size) in Petri dishes, with shading of treatment corresponding to the percent response bars below. The control dish (left) contains an untreated diet station (NT) paired with one treated with distilled water. The experimental dishes (right) each contain an untreated diet station (NT) paired with one treated with conspecific larval oral exudate. In dish 1, exudate was from another individual (test larva not induced to produce exudate). In dish 2, exudate was from the test larva (test larva induced). In dish 3, exudate was from another individual (test larva induced). $N = 40$. With no delay in introduction of test larva, only the distribution in dish 1 is significantly different from the control, Fisher's exact test, $P = 0.032$. Following a 24-hr delay, all experimental distributions are significantly different from the control, $P = 0.025$, $P = 0.043$, and $P = 0.033$, respectively.

proprioception of the partially empty gut. Such a mechanism would enable larvae to use exudate to repel competitors from valuable resources, such as silken feeding tunnels (Berenbaum et al., 1993), without repelling themselves. Under field conditions, a refractory period of 24 hr may be sufficient for most of the active components in the exudate to volatilize, degrade, or be washed away. However, in the laboratory, some larvae that had been induced to produce exudate in or at the mouths of their own feeding tunnels abandoned the tunnels 6–8 hr later (Poirier and Borden, 1995). This dispersal may reflect a natural response in which the persistent occurrence of exudate (whether self- or otherwise produced) could signal an overpopulated habitat.

Many avenues of investigation remain to be explored. These include determination of the role of natural host foliage in the production and potency of the exudate, the persistence and chemical nature of the exudate, and evaluation of the potential utility of the exudate or its components in the management of spruce budworms.

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SYSTEMIC INDUCTION OF FEEDING DETERRENTS IN COTTON PLANTS BY FEEDING OF *Spodoptera* SPP. LARVAE

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Abstract—Cotton, *Gossypium hirsutum* L., has been shown to exhibit systemic induced resistance to arthropods under certain conditions. We conducted experiments to determine the effects of previous feeding of *Spodoptera exigua* Hübner and *Spodoptera littoralis* (Boisd.) larvae on feeding behavior, growth, and survival of larvae subsequently feeding on cotton. In one feeding choice test, *S. exigua* larvae preferred young leaves from undamaged control plants to undamaged young leaves from a previously damaged plant. Feeding deterrence was noticeable after only 6 hr of initial feeding damage by larvae, and there was almost complete deterrence after 30 and 54 hr of continuous feeding. In a second feeding choice test, *S. littoralis* larvae fed more on mature leaves from undamaged control plants than on undamaged mature leaves from previously damaged plants. In no-choice tests, third instars of *S. littoralis* fed undamaged young leaves from damaged plants did not gain weight and died by the seventh day, whereas larvae fed young leaves from undamaged control plants gained weight and pupated within 11 days. Sixth instars of *S. littoralis* fed either old damaged leaves, old undamaged leaves, or young undamaged leaves all from previously damaged plants gained weight slowly and took more than 12 days to pupate, whereas larvae fed young leaves from undamaged plants gained weight rapidly and pupated within five days of the beginning of the experiment.

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INTRODUCTION

Plants have evolved many traits, both physical and chemical, that protect them from damage by herbivores. Resistance may be either constitutive, when resistance factors are present regardless of injury, or induced. Induced resistance is the qualitative or quantitative enhancement of a plant's defense mechanism in response to physical or chemical extrinsic stimuli, such as herbivory, pathogen infection, or mechanical injury (Kogan and Paxton, 1983). Induced responses to injury may be localized (i.e., restricted to a small area around the actual site of damage), or systemic, where a signal produced at the damaged site is transported throughout the plant causing effects at sites distant from the initial injury. While much is known about constitutive plant resistance to arthropods, relatively less is known about induced resistance, especially systemic induction of resistance. This emerging area of plant-insect interactions is gaining considerable interest (Karban and Myers, 1989; Tallamy and Raupp, 1991; Schultz, 1993).

Cultivated cotton, *Gossypium hirsutum* L., has been studied intensively for resistance to important arthropod pests such as corn earworm, *Helicoverpa zea* Boddie; tobacco budworm, *Heliothis virescens* (F.); boll weevil, *Anthonomus grandis* Boheman; spider mites; and other pests (Niles, 1980). Constitutive factors contributing to resistance to lepidopterous pests include morphological traits, such as foliar form, color, and pilosity (reviewed in Niles, 1980), and the presence of allelochemicals, such as sesquiterpenes (Stipanovic et al., 1986), terpenoid aldehydes (Stipanovic et al., 1986; Hedin et al., 1992), condensed tannins, and flavonoids (reviewed in Hedin et al., 1983).

Localized induction of resistance to *Spodoptera littoralis* Boisd. was found in cotton leaves that experienced artificial wounding (Croxford et al., 1989). Larvae avoided feeding at the edge of the wound and in a halo surrounding the damaged site. The degree of avoidance increased up to seven days after damage and decreased with distance up to 1 cm from the wound. Systemic resistance in cotton to spider mites, *Tetranychus urticae* Koch, was induced by feeding damage on the cotyledons (Karban and Carey, 1984). Mite-exposed plants were less suitable for mite population increase than plants never exposed to mites. In addition, female mites showed a slight preference for unexposed plants over previously exposed plants in choice situations (Harrison and Karban, 1986). Beet armyworm, *Spodoptera exigua* Hübner, had a lower probability of survival from second instar to pupation on cotton plants previously exposed to mites but did not orient towards nor consume more of the foliage from unexposed plants than from previously exposed plants (Karban, 1988).

In general, studies to date on herbivore-induced resistance have produced variable results, and the effects on herbivore performance observed have often been slight (Fowler and Lawton, 1985). In the experiments reported here, we used cotton and two lepidopterous herbivores, *S. exigua* and *S. littoralis*, to determine the subsequent palatability and suitability for development of foliage from plants previously damaged by larval feeding. In particular, we wanted to determine whether systemic feeding deterrence could be induced by previous larval feeding and how quickly such a response would be noted. Secondly, would both young and mature leaves be similarly affected? Finally, would plant response to previous feeding influence both larval feeding choice and survival?

METHODS AND MATERIALS

Plants. Cotton plants (var. DPL 90) were grown in 16-cm-diameter pots filled with a mixture of potting soil and vermiculite (3 : 1) in either a greenhouse or an environmental chamber. The greenhouse was illuminated with natural light, and conditions were ambient for Florida summer (14L : 10D light cycle, 85 ± 10% relative humidity, and 30 ± 10°C). Environmental chamber conditions mimicked conditions in the Florida greenhouse (14L : 10D light cycle, 30/20°C, 75/90% relative humidity (day/night), and ca. 360 μmol photons/sec/m^2 at 400–700 nm). Plants were watered as necessary and fertilized once a week with a complete liquid fertilizer (5-1-4 with micronutrients). Plants used in experiments were at the six- to eight-true-leaf stage (one month to six weeks after planting).

Insects. Spodoptera exigua larvae were reared on an artificial diet, based on pinto beans, according to established methods (King and Leppla, 1984). *Spodoptera littoralis* larvae were reared on a potato-based artificial diet (Hinks and Byers, 1976). Larvae were starved for 24 hr prior to all experiments to ensure immediate feeding during bioassays. Larvae were used once in a bioassay and then discarded.

Feeding Choice Bioassays. Two feeding choice experiments were conducted. The first was designed to determine whether feeding by third instars on mature cotton leaves would deter subsequent feeding on young developing leaves. The second was designed to determine whether feeding on mature foliage would deter subsequent feeding on already mature fully developed cotton leaves.

In the first bioassay, two third instars of *S. exigua* were confined on each of the two oldest true leaves of cotton plants (Figure 1A). Larvae were confined in cages made from a modified Petri dish. Each half of the Petri dish was provided with a ventilation hole covered with gauze material. A styrofoam ring cushioned each side of the cage where it touched the leaf. The two sides of the cage were held together with hair clips, and the cage was held horizontal by wooden sticks placed in the soil.

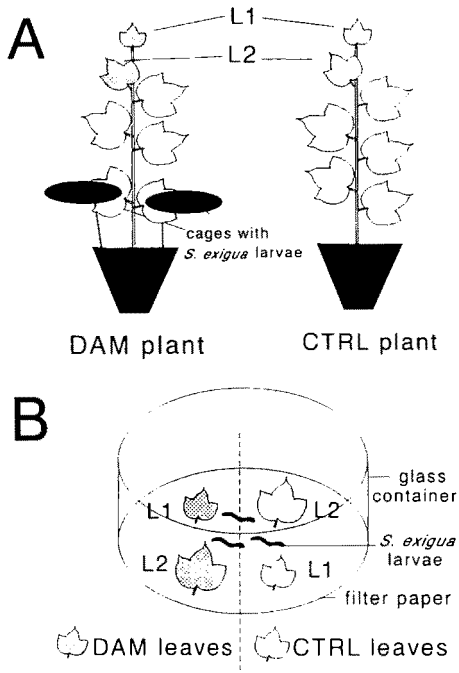

FIG. 1. (A) Experimental set-up for induction of feeding deterrents in cotton by third instars of *S. exigua*. The youngest leaves, leaf 1 (L1) and leaf 2 (L2), were cut from the damaged (DAM) and control (CTRL) plants after the oldest leaves had been exposed to larvae feeding for either 6, 30, or 54 hr. (B) Larvae were allowed to choose among leaves from control and damaged plants in a 19-cm-diameter glass arena and their consumption was measured after 18 and 24 hr.

Larvae were introduced into the cages at 11:00 hr and were replaced daily with starved third instars for up to three days. The two youngest leaves (L1 and L2, Figure 1A) were cut from one plant after 6 hr of continuous feeding, from a second plant after 30 hr of feeding, and from a third plant after 54 hr of feeding. The L1 and L2 leaves cut from the larvae-damaged plant (DAM) were paired with two leaves matched for size, age, and position within the plant from an undamaged control plant (CTRL). Preliminary experiments indicated that attaching Petri-dish cages to leaves without larvae had no effect on subsequent larval feeding preference. The four leaves were placed in a glass arena (10 cm high × 19 cm outside diameter) with three third instars placed in the center (Figure 1B). Leaf area was determined by tracing the outline of a photocopy of each leaf with a graphic digitizer (model 2200, Jandel Scientific, Sausalito,

California) before the experiment began, after 18 hr of feeding, and after 24 hr of feeding. Each time, the leaves were carefully examined for small amounts of damage that were then marked on the photocopy. The experiment was replicated five times.

In the second bioassay to determine whether mature, already fully developed leaves would be affected by larval feeding, two third instars of *S. littoralis* were caged on the oldest true leaf of five cotton plants. Larvae were removed after being allowed to feed overnight (ca. 16 hr), and plants were placed, along with undamaged control plants, in an environmental chamber for five days. After this time, the leaf immediately above the damaged leaf was caged with a leaf from the control plant matched for size, age, and position within the plant. Two third instars were added to this cage and allowed to feed overnight. After 16 hr, the leaves were cut off the plants and the area consumed was measured.

Larval Growth and Development in No-Choice Feeding Bioassays. Feeding damage in experimental plants was produced by caging one egg mass of *S. littoralis* on each of the two oldest leaves of a cotton plant (Figure 2A). When 10–20 eggs had hatched, the remaining eggs were removed and larvae were allowed to feed. After five days, all larvae were removed and the damaged plants, as well as undamaged control plants, were placed in an environmental chamber.

In the first no-choice experiment, 10 days after the larvae that caused the initial damage had been removed, young leaves that had developed after the initial damage were removed from the plant. Two third instars were placed in each of eight 9-cm-diameter Petri dishes, four containing an undamaged leaf from a previously damaged plant and four containing a leaf from a undamaged control plant. Each day fresh leaves of the same type were added, and larvae were weighed until they commenced wandering prior to pupation or died.

In a second no-choice experiment, leaves were taken from damaged and control plants 12 days after the larvae that caused the initial damage were removed. One of four types of leaves was presented to a sixth instar in a Petri dish: (1) old damaged leaves (OD) or (2) old nondamaged leaves (OND), both from damaged plants; (3) young nondamaged leaves (YND) that developed on damaged plants after the initial feeding damage occurred; or (4) young nondamaged leaves from nondamaged control plants (CTRL) (Figure 2B). Nine replicates of the four leaf types were set up. Leaves were replaced daily and larvae were weighed until they died or commenced wandering in search of pupation sites.

Statistical Analyses. For choice tests, the amount of foliage eaten from control or damaged cotton plants was expressed as a proportion of total leaf area consumed within the choice arena. Data were analyzed by analysis of variance. For no-choice tests, significant differences in larval weights were tested by paired t tests or Tukey's studentized range tests after analysis of variance,

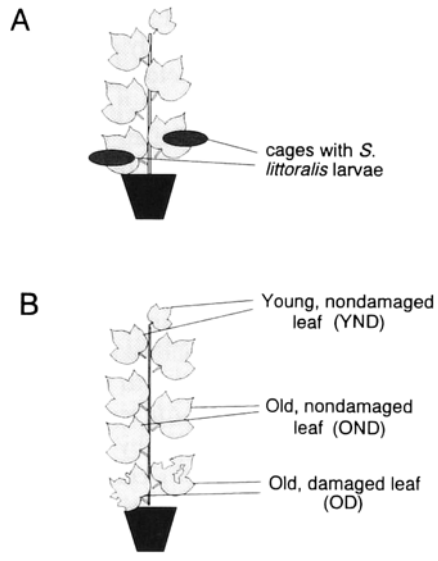

FIG. 2. (A) Experimental set-up for induction of feeding deterrents in cotton by first instars of *S. littoralis*. Initial damage was produced by caging one egg mass on each of the two oldest leaves. After eggs had hatched, larvae were allowed to feed for five days and were then removed. (B) Larval feeding response and growth were determined 10 or 12 days after larvae were removed. Larvae were presented either old, damaged leaves (OD), old, nondamaged leaves (OND), or young, nondamaged leaves (YND) from previously damaged plants, or young, nondamaged leaves from undamaged control plants (CTRL).

using a significance level of 5%. All analyses were performed with SYSTAT (Systat Inc., Evanston, Illinois).

RESULTS

Feeding Choice Bioassays. In the first bioassay, feeding by third instars of *S. exigua* on mature cotton leaves deterred feeding by subsequent larvae on young developing leaves, indicating systemic induction of feeding deterrents in developing leaves. At least 50% of the area of each type of leaf remained in all replicates of the experiment. Therefore, leaf consumption was expressed as the percentage of total leaf area eaten in a replicate for each combination of leaf age and treatment (Table 1).

Treatment contributed significantly to the variation in percentage consumption of foliage for all durations of initial feeding damage and at both

TABLE 1. LEAF AREA CONSUMED AFTER 18 AND 24 HOURS FROM YOUNGEST (L1) AND SECOND YOUNGEST (L2) LEAVES OF UNDAMAGED COTTON PLANTS (CTRL) AND PLANTS PREVIOUSLY DAMAGED BY 6, 30, AND 54 HOURS OF FEEDING BY THIRD INSTARS OF *S. Exigua* CONFINED ON TWO OLDEST LEAVES (DAM)

Duration of initial feeding damage (hr)	Treatment	18 hr feeding		24 hr feeding	
		L1	L2	L1	L2
Percentage of total leaf area consumed (mean ± SEM)					
6	DAM	1.38 ± 0.88	30.68 ± 10.65	3.90 ± 3.36	32.99 ± 7.54
	CTRL	7.36 ± 5.25	60.58 ± 8.29	8.67 ± 5.38	54.46 ± 7.65
30	DAM	0.10 ± 0.06	16.58 ± 9.39	0.46 ± 0.12	12.64 ± 4.83
	CTRL	5.05 ± 1.78	78.27 ± 10.42	4.91 ± 2.00	81.99 ± 5.75
54	DAM	0.84 ± 0.84	2.21 ± 2.21	0.64 ± 0.60	2.38 ± 2.09
	CTRL	13.55 ± 8.05	83.4 ± 8.38	13.68 ± 6.72	83.30 ± 6.61
Leaf area consumed (mm^2) (mean ± SEM)					
6	DAM	14 ± 6	454 ± 183	51 ± 39	787 ± 207
	CTRL	154 ± 126	999 ± 242	248 ± 192	1353 ± 309
30	DAM	2 ± 1	124 ± 43	7 ± 2	252 ± 154
	CTRL	57 ± 25	1176 ± 322	74 ± 27	1581 ± 338
54	DAM	3 ± 3	7 ± 7	4 ± 4	17 ± 13
	CTRL	178 ± 137	933 ± 233	227 ± 127	1313 ± 296

observation periods (18 or 24 hr) (Table 2). As little as 6 hr of feeding damage inflicted by third instars of *S. exigua* caused subsequent larvae to consume a significantly smaller percentage of foliage from previously damaged plants than from control plants (Table 1) ($P = 0.025$ at 18 hr and $P = 0.052$ at 24 hr; Table 2). Young leaves from plants experiencing 30 and 54 hr of feeding injury were significantly less preferred than leaves from control plants (Tables 1 and 2). The duration of initial feeding damage significantly influenced percentage consumption of both control ($F = 4.54$; $df = 1,16$; $P = 0.049$) and damaged leaves ($F = 6.19$; $df = 1,16$; $P = 0.024$) measured after 24 hr of larval feeding. As duration of initial feeding injury increased (from 6 to 54 hr) leaves from previously damaged plants contributed less to the total consumption, while leaves from control plants contributed correspondingly more (Table 1).

Leaf age (the youngest leaf, L1, or the second youngest leaf, L2) was a significant source of variation for all combinations (Table 2). Larvae always consumed smaller amounts of the youngest leaves (L1), regardless of whether they were from control or previously damaged plants (Table 1). There was also a significant interaction between leaf age and treatment for all time periods except for feeding injury of 6 hr (Table 2). This can be explained by the observation that while L2 leaves from control plants were very much more palatable

TABLE 2. ANALYSIS OF VARIANCE OF PERCENTAGE OF LEAF AREA CONSUMED BY THIRD INSTARS OF *S. Exigua* IN 18 OR 24 HOURS IN CHOICE TESTS AFTER INITIAL FEEDING DURATION OF 6, 30, OR 54 HOURS

Duration of initial feeding damage (hr)	Source of variation	*df*	18 hr feeding			24 hr feeding		
			Mean squares	*F*	*P*	Mean squares	*F*	*P*
6	Leaf[a]	1	8510.53	32.36	<0.001	7009.26	36.04	<0.001
	Treatment[b]	1	1608.90	6.12	0.025	860.42	4.24	0.052
	Leaf × treatment	1	714.70	2.72	0.119	348.25	1.79	0.200
	Error	16	263.01			194.49		
30	Leaf	1	10059.74	40.26	<0.001	9961.17	131.90	<0.001
	Treatment	1	5552.54	22.22	<0.001	6806.69	90.13	<0.001
	Leaf × treatment	1	4024.26	16.01	0.001	5264.17	69.70	<0.001
	Error	16	249.89			75.52		
54	Leaf	1	6341.30	36.06	<0.001	6363.43	54.44	<0.001
	Treatment	1	11023.97	62.68	<0.001	11034.66	94.40	<0.001
	Leaf × treatment	1	5861.72	33.33	<0.001	5759.66	49.27	<0.001
	Error	16	175.87			116.90		

[a] Youngest (L1) or second youngest (L2) leaf.
[b] Undamaged control (CTRL) or previously damaged (DAM) plants.

than L2 leaves from damaged plants, L1 leaves from control plants were not much more palatable than these youngest leaves from damaged plants (Table 1). The youngest leaves appeared to be inherently protected against herbivore attack.

Results from the second bioassay indicated that previous feeding damage by third instars of *S. littoralis* induced production of feeding deterrents in already mature, nondamaged leaves (the leaf immediately above the damaged leaf). When *S. littoralis* larvae were confined with still-attached leaves from damaged and undamaged plants, 99.1 ± 0.9% (mean ± SD, $N = 5$) of the leaf area consumed was from the control rather than the previously damaged plant.

Larval Growth and Development in No-Choice Feeding Bioassays. Third instars of *S. littoralis* confined with young undamaged leaves from a previously damaged cotton plant or with young undamaged leaves from a control plant differed significantly in weight gain and survival over the nine-day course of the experiment (Figure 3). Larvae presented with undamaged foliage from a previously damaged plant refused to feed and did not gain weight. All eight larvae died by day 7 with seven, five, and one larva remaining alive on days 4, 5, and 6, respectively. On the other hand, larvae fed leaves from a control plant gained weight almost exponentially after day 5. These larvae survived to day 9 of the experiment, when they left the foliage in search of pupation sites. Larvae in the two treatments weighed the same at the start of the experiment, but larvae fed control foliage weighed significantly more than larvae fed induced foliage on days 1–6 (two-tailed t test, $\alpha = 0.05$).

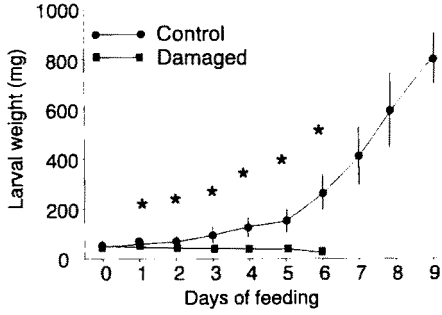

FIG. 3. Average weight (±1 SD, $N = 8$) of *S. littoralis* larvae confined in Petri dishes as third instars with young, undamaged leaves from either a damaged cotton plant (damaged, ■) or an undamaged control plant (control, ●). Larvae were weighed daily until they left the foliage in search of pupation sites (day 10 for larvae fed control leaves) or they died (day 7 for larvae fed leaves from previously damaged plants). *Significant difference in larval weight (two-tailed t test, $\alpha = 0.05$).

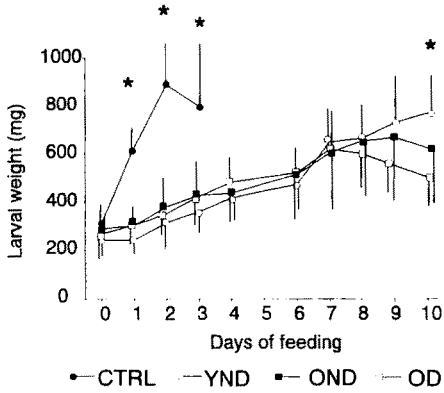

FIG. 4. Average weight (±1 SD, $N = 9$) of *S. littoralis* larvae confined in Petri dishes as sixth instars with either old, damaged leaves (OD, □), old, nondamaged leaves (OND, ■), or young, nondamaged leaves (YND, ○) from previously damaged plants, or young, nondamaged leaves from undamaged control plants (CTRL, ●). Larvae were weighed daily until they left the foliage in search of pupation sites (day 4 for larvae confined with undamaged control leaves and day 11 for all others). *Significant difference in larval weights among the four leaf types (Tukey's studentized range test, $\alpha = 0.05$).

Sixth instars of *S. littoralis* presented with one of three types of leaves from previously damaged cotton plants (YND, OND, or OD, Figure 2B) or with leaves from undamaged plants (CTRL) all attempted to feed; however, weight gain and survival differed significantly among the treatments (Figure 4). Larvae fed young foliage from control plants gained weight significantly faster than larvae fed foliage from previously damaged plants (Tukey's studentized range test, $\alpha = 0.05$) and left the foliage in search of pupation sites by day 4 of the experiment. Larval weight did not differ significantly among the other three treatments until day 10, when larvae fed young undamaged foliage from the previously damaged plant (YND) weighed more than larvae fed the old damaged leaves from the damaged plant (OD) (Figure 4). After 10 days, the induced plants were severely depleted of foliage. Therefore, remaining larvae had to be fed noninduced foliage and, as a consequence, survival to pupation and emergence could not be calculated. However, all but two of the larvae fed induced foliage for 10 days eventually pupated and produced normal-looking moths (one larva fed OD foliage died on day 6 and one larva fed OND foliage died on day 8).

DISCUSSION

Systemic induction of antifeedants in response to insect herbivory was first discovered in tomato and potato (Green and Ryan, 1972). Since then research

into systemic responses to herbivory has increased dramatically. Research efforts can be divided into those addressing annual or herbaceous perennial plants such as tomato, soybean, cucurbits, tobacco, and cotton, and studies on trees such as birch, alder, oak, willow, conifers, quaking aspen, and poplar (reviewed in Tallamy and Raupp, 1991). Differences in the defensive strategies of these two groups are to be expected because of their differing "apparency" to enemies (Feeny, 1977). Most of the accounts of rapid, systemic, induced responses to insect herbivores occur in annual plants.

In our experiments, larvae of two *Spodoptera* species fed more on foliage from undamaged cotton plants than on undamaged foliage from plants previously fed upon by larvae. In contrast, Karban (1988) found that *S. exigua* larvae did not consume more foliage from undamaged cotton plants than foliage from plants exposed to mites at the cotyledonary stage when given a choice. Female mites, however, were observed to choose foliage from unexposed plants over foliage from previously mite-exposed plants for feeding and oviposition (Harrison and Karban, 1986). They suggested that the mites made this choice without contacting the plant or feeding, and instead relied on olfactory cues. While we know that volatiles produced systemically from undamaged leaves of herbivore-damaged cotton plants differ from volatiles released from undamaged plants (Röse et al., unpublished data), we do not know whether volatile signals influenced the feeding choices of *S. exigua* or *S. littoralis*.

In studies by other researchers, *S. littoralis* avoided the immediate area around a disk punched in cotton and soybean leaves, indicating some localized antifeedant (Croxford et al., 1989). The soybean looper, *Pseudoplusia includens* (Walker), and *Epilachna varivestis* Mulsant, the Mexican bean beetle, fed preferentially on disks cut from foliage of uninjured soybean rather than on disks from undamaged foliage of plants previously fed on by soybean looper (Lin and Kogan, 1990). *Trichoplusia ni* (Hübner) consumed more low-alkaloid content tobacco leaves than high-alkaloid content leaves (Baldwin, 1991) when alkaloids had been induced by mechanical damage. *S. littoralis* fed more on tomato leaves from undamaged plants than on damaged or undamaged leaves from a previously wounded plant (Edwards et al., 1985).

Results from the preceding experiments indicate that many herbivores are capable of choosing to feed on noninduced foliage rather than on induced foliage. For mobile insects, such as lepidopterous larvae, avoidance behavior in response to antifeedants may result in mortality in the field due to increased encounters with predators, pathogens, or parasitoids; falling off the plant; or expenditure of energy searching for suitable feeding sites rather than feeding. The relationship between laboratory bioassays and population effects in the field is not often explored, however (Fowler and Lawton, 1985; Karban, 1991b).

In our experiments, feeding by two third instars of *S. exigua* on the two oldest cotton leaves produced pronounced systemic feeding deterrency in the two youngest leaves in as little as 6 hr (Table 1). The factor responsible for

deterrence was somehow translocated upwards and its effects were observed at least three nodes higher than the location where initial damage occurred. No research to date has addressed the speed of onset of systemic induction in cotton other than that of Karban and Carey (1984), who found that feeding by mites produced noticeable effects within 14 days of initial feeding damage. In tomato, however, *S. littoralis* fed significantly less on leaves cut with scissors than on control leaves within 8 hr of injury. The same was true of the leaf adjacent to the wounded leaf 24 hr after damage (Edwards et al., 1985). Green and Ryan (1972) found that within 1 hr of artificial wounding in tomato, sufficient signal has been transported that leaves adjacent to the injured leaves were induced and contained elevated levels of proteinase inhibitors. Thus the speed of onset of systemic induction in our experiments with cotton is similar to that found by other researchers for tomato.

In our studies, we observed reduced feeding by larvae in choice tests involving both young, still-developing leaves and fully mature leaves. Similarly, larvae feeding on either mature leaves or young leaves from induced plants in no-choice situations both gained weight slowly. Presumably, fewer primary metabolites are being directed towards the mature leaves and more to the developing leaves; however, the signal still travels to the mature leaves and induces feeding deterrents or growth inhibitors. The distribution of induced responses within plants has not been studied in much detail (Karban and Myers, 1989) and will be addressed in future research.

The detrimental effects to larvae of feeding on foliage from previously damaged plants were significant and pronounced in our experiments. Third instars of *S. littoralis* refused to feed on foliage from induced plants. Larvae lost weight during the experiment and all died by day 7, whereas larvae fed foliage from undamaged plants gained weight rapidly and pupated successfully. Sixth instars confined on induced foliage during their period of almost exponential weight gain also gained weight significantly more slowly than those larvae confined on young foliage from control plants. Larvae fed either damaged old leaves, undamaged old leaves, or young leaves, all from previously damaged plants, did not differ significantly in weight until the last day of the experiment (day 10, Figure 4), when larvae fed young leaves weighed more than larvae fed either type of old leaves.

In general, herbivores that feed on artificially wounded or herbivore-damaged plants often exhibit reduced population growth, survival, growth rates, adult weight, and adult fecundity (reviewed in Karban, 1987) compared to herbivores feeding on undamaged plants. For example, *S. exigua* reared on foliage from mite-exposed cotton plants had a lower probability of surviving from second instars to pupation and reduced expected life-spans compared to larvae fed control plants (Karban, 1988). Population growth of *T. urticae* on previously mite-exposed cotton plants was about half that on unexposed plants (Karban and

Carey, 1984). Localized induced resistance in a wild cotton, *Gossypium australe*, led to decreased survival of the leafminer, *Bucculatrix gossypii*, on leaves previously damaged by leafminers (Karban, 1991a). The detrimental effects on herbivores observed in these experiments are slight compared to results of our studies.

Many questions arise from these experiments, and this model system should provide fertile ground for further research. It is not known whether the induced response we observed was chemical or physical in nature. Known antixenotic and antibiotic allelochemicals conferring constitutive resistance to herbivores of cotton may be inducible, and this should be investigated. Similarly, is the induction of feeding deterrents related to the induction of volatiles in cotton? In corn seedlings, mechanical damage alone does not induce systemic production of volatile sesquiterpenes (Turlings et al., 1990). Larval feeding damage or application of larval regurgitant to mechanical damage is necessary for induction of volatiles. Comparative studies on the effects of mechanical damage versus larval feeding damage on feeding deterrents in cotton will address this issue. Finally, cotton leaves were not sterilized prior to the experiments, so it is possible that phylloplane microbes elicited the responses observed rather than insect-related factors (Benedict and Chang, 1991). Few experiments, in fact, separate these two factors (Schultz, 1993). Well-designed experiments could shed light on the interesting phenomenon of systemic induced resistance in cotton.

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USE OF A COMPARATIVE APPROACH TO IDENTIFY ALLELOPATHIC POTENTIAL AND RELATIONSHIP BETWEEN ALLELOPATHY BIOASSAYS AND "COMPETITION" EXPERIMENTS FOR TEN GRASSLAND AND PLANT SPECIES

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Abstract—Various allelopathy bioassays were used to evaluate the allelopathic potential of 10 grassland forage species against a common test (phytometer) species, *Carduus nutans* L. Aqueous extracts did not influence *C. nutans* germination, although radicle elongation was often severely inhibited. *C. nutans* was strongly affected by shoot, but not root, leachates. Decomposing ground tissue had mixed effects, and often stimulated shoot production of *C. nutans*. Calculation of R^2 (coefficient of determination) values between these results, and the results of previous experiments investigating the effects of the same 10 species on *C. nutans* emergence and development in field plots and glasshouse competition experiments frequently revealed strong, statistically significant relationships. Our results therefore provide correlative evidence for the importance of allelopathy in field conditions.

Key Words—Allelopathy, bioassay, *Carduus nutans* L., comparative approach, correlative evidence, forage plant, grassland, phytometer.

INTRODUCTION

Allelopathy has frequently been suggested as an important factor in regulating the structure of plant communities (McPherson et al., 1971; Rice, 1984; Rutherford and Powrie, 1993; Smith and Martin, 1994) and growth rate of plants in

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field conditions (Dias, 1991; Li et al., 1992; Nakahisa et al., 1993). However, many researchers have criticized the notion that allelopathic agents are active or important under field conditions, mainly because of perceived flaws in the methodologies (principally bioassays) used for evaluating allelopathy (Harper, 1977; Keeley, 1988) and because of the failure of one study (Stowe, 1979) to detect a statistically significant relationship between the bioassay results for a range of species and the spatial patterning of the same species in the field.

Comparative approaches to plant ecology often have been utilized successfully for testing hypotheses relating to factors influencing plant growth and structuring plant communities (Gaudet and Keddy, 1988; Grime et al., 1988; Shipley and Peters, 1990; Keddy et al., 1994). Such approaches involve selecting a number of plant species (with each species representing a single independent data point), and investigating whether a statistically significant relationship (usually a correlation) exists between two or more variables measured for the whole range of species tested. Although correlation does not mean causation, predictive comparative approaches can enable prediction of factors important in regulating a wide spectrum of species in a given community (Peters, 1991).

The comparative approach has considerable potential in testing the allelopathy hypothesis. Such a test would involve quantifying the relative allelopathic effect of a range of donor species against a common receiver species (= phytometer; Gaudet and Keddy, 1988) and determining whether these effects were correlated with the relative effects of the same donor species against the receiver species in field (or glasshouse) interference experiments. In previous studies we have reported the relative effects of 10 grassland forage plant species against the seedling emergence, growth, and development of a single test species (the pasture weed *Carduus nutans* L. or nodding thistle) in both field experiments (Wardle et al., 1995) and glasshouse competition experiments (Wardle et al., 1992b). The purposes of the present study were to utilize various conventional bioassay techniques to determine the potential allelopathic effects of the same 10 species on *C. nutans* and to employ the comparative approach to elucidate whether significant relationships could be established between the results of these bioassays and the results of Wardle et al. (1992b, 1995).

METHODS AND MATERIALS

The donor species used for this study are all used as forage species in New Zealand pastures, and included six grasses [*Dactylis glomerata* L. (cocksfoot), *Phalaris aquatica* L. (phalaris), *Bromus wildenowii* L. (prairie grass), *Lolium perenne* L. (perennial ryegrass), *Festuca arundinacea* Schreb. (tall fescue), and *Holcus lanata* L. (yorkshire fog)] and four legumes [*Medicago sativa* L.

(lucerne), *Trifolium pratense* L. (red clover), *T. subterraneum* L. (subterranean clover), and *T. repens* L. (white clover)]. Seedlings of donor species were raised from commercially available seed sources. Seeds for the receiver species, *C. nutans*, were collected from pastures in the Waikato district of New Zealand [area described in detail by Wardle et al. (1995)], during the summer of 1990–1991. Four bioassays were used to evaluate the effects of each of the 10 species on *C. nutans*: aqueous extract bioassays, leaf leachate bioassays, root leachate bioassays, and decomposing tissue bioassays.

Tissue Preparation. To provide tissues for the allelopathy bioassays, polystyrene trays, 50 × 30 × 9 cm deep were filled up to 1 cm depth with a 1 : 1 mixture of soil and pumice, as described by Wardle et al. (1992b). The soil was a Vitric hapludand (Horotiu sandy loam; pH 5.7; organic C 9.1%); the pumice was included to ease the separation of roots. These trays were sown with seeds of each of the 10 species (eight trays per species) at the recommended field sowing rate for each species [rates given by Wardle et al. (1992b)]. Following establishment, the swards were left in glasshouse conditions (temperature range: 10–25°C; day–night light ratio 12 : 12) and amended approximately every two weeks with an appropriate nutrient solution (Smith et al., 1983). After 90 days all the above-ground and below-ground tissue produced by each species was harvested.

Aqueous Extract Bioassay. This was conducted according to Wardle et al. (1992a). Subsamples of the shoot and root tissues of each species were air-dried and ground to 0.5 mm using a Wiley mill, mixed with deionized water at a concentration of 5% (w/v), and stirred vigorously at room temperature for 20 min. The mixture was filtered by passing it twice through cheesecloth and then through Whatman No. 1 filter paper. For each tissue, two solutions, 2% and 5% w/v, of tissue were prepared. These concentrations are within the range normally used for bioassays of this type (Stowe, 1979; Hegazy et al., 1990). To assess the allelopathic effect of each solution, 10 replicate 9-cm-diameter Petri dishes were set up and germination tests performed as described by Wardle et al. (1992a). Two pieces of Whatman No. 1 filter paper (9 cm diameter) were added to each dish, to which was added 5 ml of aqueous extract and 20 seeds of *C. nutans*. Controls consisted of 10 Petri dishes containing deionized water in place of the extract. Osmotically adjusted controls were not used (cf. Wardle et al., 1992a) because *C. nutans* is not responsive to osmotic potentials of the extracts. The total number of seeds that germinated for each Petri dish was determined daily for 21 days and seeds were removed upon germination so as to prevent radicles and cotyledons of germinated seeds from interfering with germination of other seeds. Total percent germination for each Petri dish was determined for the whole 21-day period, as well as the speed of germination index, *S*, of Khandakar and Bradbeer (1983) as described by Ahmed and Wardle (1994). This index ranges from 100 (if all seeds germinate on the first day

following set-up) to 0 (if no seeds germinate by the end of the experiment). Assessments of allelopathic effects of these extracts on *C. nutans* radicle elongation were also made, as described by Wardle et al. (1993). Seeds of *C. nutans* were pregerminated on moistened filtered paper, and upon germination were placed in Petri dishes (three seeds per dish) with 5 ml of each of the solutions described above and five replicate dishes per extract. Corresponding controls consisted of deionized water in place of the extract. After 10 days, the length of each radicle was measured.

Aqueous Leaf Leachate Bioassay. This bioassay was performed for each donor species as described by Wardle et al. (1993), based on the approach of McPherson et al. (1971) and Stowe (1979). This involved placing 1.5 kg (fresh weight) shoot tissue on a 1-m-diameter sieve (holes = 4 mm) and spraying it with a fine mist of deionized water for approximately 15 min, until 200 ml of leachate was obtained. The effect of each leachate on percent germination, speed of germination, and radicle elongation of *C. nutans* was evaluated exactly as for the aqueous extract bioassay.

Root Leachate Bioassay. This bioassay was performed basically as described by Ahmed and Wardle (1994). Six replicate pots (15 cm diameter × 12 cm deep) were prepared for each donor species by filling them with a 1 : 1 mixture of Horotiu silt loam soil and pumice, and sowing them with seeds of that species at the recommended sowing rate (Wardle et al., 1992b). Another six pots, filled with the soil–pumice mix but left unplanted, were set up to serve as controls. All pots were then left in glasshouse conditions as described above, and trimmed every two to three weeks to 5 cm height (to simulate grazing removal in the field; Wardle et al., 1994). After 90 days a further 66 pots were prepared, each filled with the same soil–pumice mix, and planted with 15 *C. nutans* seeds. Each *C. nutans* (receiver) pot was linked with one donor pot (so as to maintain statistical independence). Every seven days for a further 98 days, 650 ml of deionized water was added to the soil surface of each donor pot. Over the following 2 hr, 250 ml of the water that came out of the bottom of each donor pot was collected and added to the soil surface of the corresponding receiver pot. The total number of *C. nutans* seedlings that emerged in each receiver pot was counted approximately every two days for the first three weeks and approximately every five days thereafter until the end of the experiment, so as to determine the total percent seedling emergence and speed of emergence index, E (calculated the same as the speed of germination index, S). Thirty-eight days after setting up the receiver pots, the total number of *C. nutans* seedlings was thinned to one per pot, to reduce the potential for intraspecific interference (Wardle et al., 1993). At the end of the experiment, the *C. nutans* plant present in each pot was harvested and the total above-ground and below-ground biomass dry weight, and shoot–root ratio was determined following oven drying (80°C;

24 hr). The entire experiment was performed in glasshouse conditions as described above.

Decomposing Tissue Bioassay. This bioassay was performed as described by Wardle et al., (1993). For each donor species, air-dried root and shoot tissue was ground to 0.5 mm using a Wiley mill and mixed with the soil–pumice mixture described above, at each of two concentrations: 0.2% and 0.8% w/w. The concentrations used correspond with the quantities of forage plant tissues present in managed pastures (Wardle et al., 1993). Two types of controls were also used. The first consisted of pumice + soil, without amendments. The second consisted of peat moss mixed with the pumice + soil at concentrations of 0.2% and 0.8%. The rationale behind this second control is that soil organic matter levels are enhanced following addition of plant tissue (resulting in plant responses being attributable to factors other than allelopathy), and the addition of a supposedly inert medium (peat moss) in place of plant tissue is presumed to correct for changes in organic matter without inducing allelopathic effects (Stowe, 1979; Shafer and Garrison, 1986). Six replicate pots (each 15 cm diameter × 12 cm deep) were filled with soil from each tissue type × concentration combination. Fifteen seeds of *C. nutans* were planted in each pot and the total percent seedling emergence and speed of emergence determined as described for the root leachate bioassay. Pots were maintained in glasshouse conditions as described above and amended with an appropriate nutrient solution (Smith et al., 1983). After 36 days the total number of *C. nutans* seedlings in each pot was thinned to one. The experiment was then left for a further 59 days, after which the oven-dry shoot and root weight and shoot–root ratio of each seedling was determined.

Data Analysis. Data for each bioassay were calculated as treatment value divided by control value, so as to aid comparisons between bioassays (Wardle et al., 1993; Ahmed and Wardle, 1994). These data were analyzed using analysis of variance testing for donor species effects on *C. nutans*, followed by Tukey's honestly significant difference test at $P = 0.05$.

Coefficients of determination (R^2) values were calculated in order to evaluate the relationships between the allelopathy bioassay results and the results of the studies of Wardle et al. (1992b, 1995), who investigated the nature of interference of the 10 forage species on seedling emergence, growth, and development of *C. nutans*. Because of the large number of variables measured for the 10 species (especially in terms of the allelopathy bioassays), if all possible combinations between variables were tested it would be expected that a number of R^2 values would emerge as being statistically significant simply due to chance (or type 1 error). To help overcome this problem and to reduce the number of variables in the data set, the data were simplified by using principal components analysis (PCA) to identify principal components that summarized the maximal

variation present across the 10 species for each of the four bioassays (see Wardle and Parkinson, 1990). For example, the six variables relating to the aqueous extracts (germination, speed of germination, and radicle elongation data for each of the 2% and 5% concentrations of extract) were all simplified to one variable, namely the principal component maximizing variation between donor species. Only the first principal component (explaining the greatest variation) was used from each of the four bioassay types, since preliminary analysis revealed that lower-order principal components were unimportant in explaining observed trends. A fifth allelopathy variable was also used, namely the principal component explaining the maximal variation in a previous study aimed at evaluating the effects of residual soil toxicity of each of the 10 donor species against *C. nutans* (Wardle et al., 1991) (hereafter referred to as the residual soil bioassay). R^2 analysis was used to elucidate the relationship between each of the five principal components, and the effects of swards of each of the 10 forage species on *C. nutans* seedling emergence, growth rate, mortality, and flowering intensity in the field [data from Wardle et al. (1995)] and on *C. nutans* seedling emergence and growth rate in glasshouse competition experiments [data from Wardle et al. (1992b)].

In a previous study, Wardle et al. (1993) determined the response of the same 10 forage species to the allelopathic potential of *C. nutans* tissues. To determine whether any relationship exists between the allelopathic effect of these 10 species (present study) and their response to allelopathic effects of another species [i.e., whether there exists an allelopathic equivalent of the competitive hierarchy (Panetta and Randall, 1993) of plants], R^2 analysis was performed between each of the five principal components described above and the principal components derived from data presented by Wardle et al. (1993). The analyses included principal components summarizing forage plant response to *C. nutans* aqueous extracts, *C. nutans* shoot leachates, and *C. nutans* decomposing tissues.

RESULTS AND DISCUSSION

Bioassay Data. The aqueous extract bioassays showed that total germination and speed of germination of *C. nutans* was generally unresponsive to forage plant tissues at the 2% concentration (Table 1). However, shoot extracts of several species at the 5% concentration inhibited these variables, while root extracts for five of the six grasses significantly enhanced *C. nutans* speed of germination. Radicle elongation had a stronger (and consistently negative) response to shoot and root extracts, supporting other studies that suggest that radicle elongation bioassays are considerably more sensitive than those based on germination (Rice, 1984; Leather and Einhellig, 1986). Since this bioassay is most widely used to evaluate whether water-soluble inhibitors are released

TABLE 1. EFFECT OF AQUEOUS EXTRACTS OF GRASS AND LEGUME TISSUES ON GERMINATION AND RADICLE GROWTH OF *Carduus nutans*[a]

Tissue type and species	2% tissue concentration[b]			5% tissue concentration		
	TG[c]	S	RE	TG	S	RE
Shoot tissue						
D. glomerata	108.6	116.1	5.3	96.0	81.2	1.3
P. aquatica	110.3	112.5	4.4	23.0	19.1	0.0
B. wildenowii	104.1	103.0	5.2	94.5	86.4	4.8
L. perenne	112.8	116.9	30.0	85.5	86.4	5.2
F. arundinacea	111.5	109.6	5.7	54.3	53.6	0.0
H. lanata	112.5	123.7	37.1	114.0	125.3	3.8
M. sativa	113.5	95.0	21.3	55.2	39.3	5.2
T. pratense	113.2	101.5	28.4	96.0	72.3	6.7
T. subterraneum	109.0	108.2	25.5	63.7	48.4	5.8
T. repens	100.0	86.3	5.2	66.7	28.2	3.6
Tukey's h.s.d.[d]	15.1	20.6	19.7	22.3	22.1	6.4
Root tissue						
D. glomerata	107.1	111.9	64.5	108.2	125.6	3.7
P. aquatica	115.9	142.2	62.3	112.9	133.1	10.2
B. wildenowii	108.0	119.3	45.3	110.7	131.0	13.8
L. perenne	109.6	110.1	71.7	108.6	128.8	32.9
F. arundinacea	107.9	115.8	23.9	112.8	137.8	11.6
H. lanata	113.4	132.9	41.4	112.6	136.4	28.9
M. sativa	104.6	95.2	11.2	83.4	63.8	5.3
T. pratense	112.1	109.8	35.6	103.7	81.7	9.5
T. subterraneum	110.1	117.0	30.1	107.0	105.5	11.3
T. repens	112.8	115.5	44.5	99.0	94.7	8.5
Tukey's h.s.d.	14.1	19.7	29.7	16.7	22.4	13.6

[a] All data expressed as (treatment value/control value) × 100.
[b] Weight/volume.
[c] TG, total germination; S, speed of germination index; RE, radicle elongation rate.
[d] Tukey's honestly significant difference at $P = 0.05$.

from dried plant material (Stowe, 1979), it would appear from this bioassay that establishment of seedlings from germinated seeds is likely to be generally negatively affected by the forage species studied.

The aqueous shoot leachates of grasses did not exert strong effects on germination or radicle growth of *C. nutans* (Table 2) except for *L. perenne*, which caused a significant decrease in radicle elongation. However, all four legume species induced a statistically significant stimulation of *C. nutans* speed of germination, and *T. subterraneum* induced a significant decline in *C. nutans* radicle elongation. Since this bioassay is indicative of possible effects of rainfall

TABLE 2. EFFECT OF GRASS AND LEGUME SHOOT LEACHATES ON GERMINATION AND RADICLE GROWTH OF *Carduus nutans*[a]

Species	TG[b]	S	RE
D. glomerata	106.2	105.5	83.0
P. aquatica	104.2	105.2	88.6
B. wildenowii	102.1	102.7	98.6
L. perenne	94.9	98.8	64.8
F. arundinacea	105.8	106.0	82.4
H. lanata	104.3	100.1	76.7
M. sativa	112.4	142.7	119.7
T. pratense	103.5	123.3	94.4
T. subterraneum	99.6	135.5	67.7
T. repens	101.1	129.8	110.6
Tukey's h.s.d.[c]	13.2	19.2	31.9

[a] All data expressed as (treatment value/control value) × 100.
[b] TG, total germination; S, speed of germination index; RE, radicle elongation rate.
[c] Tukey's honestly significant difference at $P = 0.05$.

passing through the leaves of forage plants and reaching target plants situated beneath them (McPherson et al. 1971), it appears unlikely that this mechanism is consistent with suppression of *C. nutans* seedlings by forage species in the field.

There were no significant effects of root leachates on *C. nutans* seedling emergence (Table 3), probably indicative of the high variability of the data. However, both shoot growth and root growth of *C. nutans* were significantly influenced by most of the forage species, with the strongest effects being shown by *P. aquatica*, *L. perenne*, *H. lanata*, and *F. arundinacea*. Nutrient analysis of selected leachates from the donor pots demonstrated that the ammonium, nitrate, and phosphate concentrations did not differ between species (data not shown), so the differential levels of inhibition induced by different species appears to be due to allelopathic, not nutrient depletion effects. For five of the 10 donor species, there was also a significant reduction of *C. nutans* shoot–root ratio, indicating that shoot growth is more susceptible to root leachate toxicity than is root growth. Although this finding is inconsistent with some previous studies (Wardle et al., 1993; Nilsson, 1994), allelopathy involves a wide range of compounds, all of which affect target plants in different ways, so perhaps differences between the results from different studies are not surprising.

The decomposing shoot and root tissues of the donor species did not significantly alter *C. nutans* seedling emergence or rate of emergence (Table 4). However, statistically significant stimulations of *C. nutans* root growth (relative

TABLE 3. EFFECT OF GRASS AND LEGUME ROOT LEACHATES ON EMERGENCE AND GROWTH OF *Carduus nutans*[a]

Species	TE[b]	SE	S	R	S/R
D. glomerata	112.4	84.2	43.2	65.9	66.9
P. aquatica	112.3	89.5	41.1	65.7	64.8
B. wildenowii	114.2	85.0	57.3	76.9	75.8
L. perenne	66.2	53.6	35.9	55.8	67.7
F. arundinacea	71.2	58.0	35.7	53.9	68.2
H. lanata	108.2	87.5	37.9	60.0	64.2
M. sativa	99.7	83.5	82.3	95.8	86.3
T. pratense	99.9	77.4	69.9	80.1	88.2
T. subterraneum	120.3	94.0	52.6	67.3	84.7
T. repens	120.7	95.6	70.5	84.6	84.3
Tukey's h.s.d.[c]	66.9	49.7	17.4	27.2	34.5

[a] All data expressed as (treatment value/control value) × 100.
[b] TE, total emergence of seedlings; SE, speed of emergence index; S, final shoot weight; R, final root weight; S/R, shoot-root ratio.
[c] Tukey's honestly significant difference at $P = 0.05$.

to the unamended controls) were caused by shoot tissues of *D. glomerata* (0.2% concentration), *P. aquatica* (0.2% and 0.8%), *B. wildenowii* (0.2%), *F. arundinacea* (0.2%), and *H. lanata* (0.2 and 0.8%), and by root tissues of *P. aquatica* (0.8%) and *F. arundinacea* (0.8%). However, none of these stimulations were statistically significant when compared with the peat moss-amended control. This suggests that the stimulation of shoot growth may not have been due to allelopathic growth stimulation (Rice, 1986) but rather due to the improvement of soil quality due to organic matter enrichment. Amendment of soil by these tissues could conceivably increase plant growth through enhancing microbial mineralization of plant-available nutrients and enhancing turnover of the soil microbial biomass (Singh et al., 1989; Wardle, 1992).

The bioassay results in combination suggest that different forage plant species may differ in relation to their effects on *C. nutans* establishment. These effects are, however, not consistent across bioassays, probably because aqueous extract and leachate bioassays assess the potential of material to release soluble compounds over a comparatively short period, while decomposition-based bioassays evaluate longer-term processes that affect plant growth and may involve soil microorganisms.

Correlative Evidence for Allelopathy. The coefficients of determination (R^2) values between the principal component scores for the 10 forage plant species (based on principal components summarizing the data for each of Tables 1–4) and the effects of the same 10 species on *C. nutans* in field plots were often

TABLE 4. EFFECT OF GRASS AND LEGUME TISSUE MIXED WITH SOIL ON EMERGENCE AND GROWTH OF *Carduus nutans*[a]

Tissue type and species	0.2% tissue concentration[b]					0.8% tissue concentration				
	TE[c]	SE	S	R	S/R	TE	SE	S	R	S/R
Shoot tissue										
D. glomerata	103	111	151	186	81	131	156	184	119	155
P. aquatica	103	114	172	164	105	91	88	250	176	142
B. wildenowii	114	107	155	165	94	137	146	166	127	131
L. perenne	114	132	147	146	101	92	93	232	164	141
F. arundinacea	117	118	157	184	85	148	143	157	89	176
H. lanata	143	150	153	152	102	111	110	213	136	157
M. sativa	95	103	130	174	75	77	79	126	184	68
T. pratense	69	70	61	55	111	69	65	51	58	88
T. subterraneum	82	80	92	101	91	95	96	72	102	71
T. repens	87	85	76	98	78	74	72	77	110	70
Peat moss	105	113	127	156	81	128	116	128	154	83
Tukey's h.s.d.[d]	56	62	48	80	39	54	53	93	93	33
Root tissue										
D. glomerata	120	125	120	148	81	128	143	145	142	102
P. aquatica	100	103	132	147	90	137	155	180	171	105
B. wildenowii	160	166	88	92	96	148	150	126	125	101
L. perenne	94	99	89	94	94	80	79	86	89	97
F. arundinacea	117	129	104	103	101	123	114	172	173	99
H. lanata	114	127	82	77	106	126	133	91	94	97
M. sativa	105	110	108	158	68	72	68	118	148	80
T. pratense	90	93	105	116	91	64	67	97	128	76
T. subterraneum	85	81	132	195	68	59	67	112	150	75
T. repens	80	77	93	99	94	36	38	105	119	88
Peat moss	105	113	127	156	81	128	116	128	154	83
Tukey's h.s.d.[d]	50	66	55	82	30	60	67	62	80	30

[a] All data expressed as (treatment value/control value) × 100; controls used for this calculation are unamended.
[b] Weight/weight.
[c] TE, total emergence of seedlings; SE, speed of emergence index; S, final shoot weight; R, final root weight; S/R, shoot–root ratio.
[d] Tukey's honestly significant difference at $P = 0.05$.

very highly significant (Table 5). This result provides strong support for the likelihood of allelopathy occurring in the field experiment of Wardle et al. (1995), in that over 50% of the variation in *C. nutans* seedling mortality (i.e., those *C. nutans* plants that died before flowering) and the proportion of *C. nutans* plants that flowered as annuals (i.e., those that reached sufficient size in their first year to flower rather than persist as biennials) in field plots could be explained by each of the four bioassays. Further, the decomposing tissue

TABLE 5. COEFFICIENTS OF DETERMINATION (R^2) FOR RELATIONSHIPS BETWEEN PRINCIPAL COMPONENTS SUMMARIZING DATA FOR ALLELOPATHY BIOASSAYS OF TEN FORAGE SPECIES AGAINST *Carduus nutans* AND RESPONSE OF *C. nutans* TO SAME 10 SPECIES IN FIELD PLOTS AND GLASSHOUSE COMPETITION EXPERIMENTS

Experiment	Response variable for *C. nutans* in experiment	Principal component summarizing variation for bioassays of: Aqueous extracts	Aqueous shoot leachates	Aqueous root leachates	Decomposing tissues	Residual soil[a]
Field experiment (Wardle et al., 1995)	mortality in first year[b]	0.638**[c]	0.518*	0.717**	0.625**	0.286
	total mortality	0.644**	0.517*	0.701**	0.659**	0.292
	proportional annuals[d]	0.774***	0.544*	0.687**	0.696**	0.373
	capitula number per plant	0.497*	0.267	0.332	0.806***	0.033
	maximum diameter (rosette)[e]	0.231	0.114	0.364	0.699**	0.033
	maximum diameter (flowering)	0.404*	0.192	0.350	0.756**	0.063
	seedling emergence[f]	0.483*	0.273	0.284	0.416*	0.117
Glasshouse experiment (Wardle et al., 1992)	shoot weight	0.508*	0.275	0.598**	0.676**	0.510*
	root weight	0.629**	0.426*	0.652**	0.677**	0.514*
	shoot–root ratio	0.018	0.066	0.012	0.305	0.029
	plant diameter	0.729**	0.535*	0.784**	0.597*	0.474*
	seedling emergence	0.623***	0.452*	0.452*	0.238	0.578*

[a] Data from Wardle et al., (1991).
[b] All field experiment data except seedling emergence is based on a labeled cohort of *C. nutans* seedlings in plots previously sown with each of the 10 donor species.
[c] *·**·*** = R^2 is significantly different to 0 at $P = 0.05$, 0.01, and 0.001, respectively (for 8 df).
[d] Proportion of labeled plants that flowered as annuals.
[e] Maximum diameter of rosette plant immediately prior to flowering.
[f] Principal component summarizing emergence data collected every 2 to 3 months for $2\frac{1}{2}$ years.

bioassay also explained over 80% of the variation in capitula number produced per *C. nutans* plant across the 10 sward types in the field, suggesting that allelopathy is important in regulating the final size that *C. nutans* plants reach when they flower and, hence, their potential reproductive fitness (or output). Essentially, the same pattern was observed when the results of the four bioassays were compared with the effects of the same 10 species on *C. nutans* in glasshouse competition experiments [data from Wardle et al. (1992b)]—*C. nutans* shoot weight, root weight, plant diameter, and seedling emergence all showed a consistent relationship with each of the four principal components. The principal component summarizing the results of the soil toxicity bioassay study of Wardle et al. (1991) did not, however, show strong relationships with either the field or glasshouse competition experiments.

It should be noted that for two of the bioassays (aqueous extract and decomposing tissue), some donor species stimulated *C. nutans*, while the same species inhibited *C. nutans* in the studies of Wardle et al. (1992b, 1995). However, in competition experiments, both allelopathy and resource competition can occur simultaneously (see Nilsson, 1994), and it could be expected that resource competition by forage species suppresses *C. nutans* growth, so that the overall effect of forage species on *C. nutans* would be considerably more negative than would be predicted by an allelopathy bioassay.

One of the principal criticisms of the allelopathy hypothesis is the supposed failure of bioassay results to correlate with those of field studies or observations [interestingly, the same demands are not usually made for experiments testing for resource competition; see discussion by Williamson, (1990)]. Moreover, the failure of Stowe (1979) to detect a statistical relationship between allelopathy bioassays for seven species and the field patterning of the same seven species is often used as evidence against the allelopathy hypothesis (see, e.g., Keeley, 1988). Our results differed from those of Stowe (1979) in that we were able to detect a statistical relationship; however, this may be due to important differences between the two studies. In our study, the experimental field plots consisted of comparatively pure swards of each of the 10 species, enabling allelopathic compounds of only one species to dominate in each plot. It is therefore possible that this accumulation of allelopathic components can be simulated in a bioassay if an appropriate concentration of tissue is used. Stowe's field site, in contrast, consisted of a diverse range of species, and it is unlikely that any one species would ever reach sufficient dominance for its allelochemicals to dominate subsequent interactions. We propose that effective and consistent allelopathic inhibition of one species by another is more likely to occur in communities of low species richness where the nature of the soil biochemistry is likely to be determined by a dominant plant species. Christensen and Muller (1975) detected a statistically significant relationship between the differential response of 10 herbaceous species to the chaparral shrub *Adenostema fasciculata*

H.&A. in field conditions and their response to the allelopathy bioassays of *A. fasciculata*. Their experiment also supports our hypothesis, since individual *A. fasciculata* shrubs would be much larger than individuals of the other species present and would therefore be more likely to have a dominating effect on the soil biochemical composition. We are aware that correlation does not mean causation, but we believe that our results help to refute one of the main criticisms of allelopathy bioassays—that their results do not correlate well with observed vegetation patterns.

Comparison of Effects and Responses. The results of our study can also be used, via a comparative approach, to test hypotheses relating to whether species that are more allelopathic are also those that are less sensitive to allelopathic effects of other species. Values of R^2 for allelopathic effects against *C. nutans* vs. allelopathic response to *C. nutans* across the 10 forage species revealed weak and inconsistent relationships (Table 6). In studies of plant competition, it has often been demonstrated that effect and response are inversely correlated (Goldberg and Fleetwood, 1987; Miller and Werner, 1987; Panetta and Randall, 1993) and that a competitive hierarchy therefore exists. No such relationship was detected in the present study, meaning that the two components of allelopathy (effect and response) are unrelated and that there is little evidence for an allelopathic hierarchy. If such a hierarchy does not exist, it could be expected that wherever allelopathy is important in field conditions, species spatial patterning would be much less predictable than in circumstances where competition is important.

Implications for Biological Control. One of the most promising applications

TABLE 6. COEFFICIENTS OF DETERMINATION (R^2) FOR RELATIONSHIPS BETWEEN PRINCIPAL COMPONENTS SUMMARIZING BIOASSAY DATA FOR ALLELOPATHIC EFFECTS OF 10 FORAGE SPECIES AGAINST *Carduus nutans* AND RESPONSE OF SAME 10 SPECIES TO ALLELOPATHIC EFFECTS OF *C. nutans*[a]

Principal component summarizing response of forage species to *C. nutans* for bioassay of	Principal component summarizing effect of forage species on *C. nutans* for bioassays of: Aqueous extracts	Aqueous shoot leachates	Aqueous root leachates	Decomposing tissues	Residual soil[b]
Aqueous extracts	0.346	0.116	0.471*[c]	0.494*	0.241
Aqueous shoot leachates	0.436*	0.169	0.139	0.476*	0.197
Decomposing tissues	0.246	0.319	0.038	0.006	0.017

[a] Response data from Wardle et al. (1993).
[b] Data from Wardle et al. (1991).
[c] *, **, *** = R^2 is significantly different to 0 at $P = 0.05$, 0.01, and 0.001, respectively (for 8 *df*).

of allelopathy is for biological control of weeds (Altieri and Doll, 1978; Lovett, 1990). Our phytometer species, *C. nutans*, is a serious weed in many temperate regions, including New Zealand (Popay and Medd, 1990), where it aggressively competes with forage plants and reduces potential production available for livestock (Thompson et al., 1987). In our previous field study (Wardle et al., 1995) we demonstrated that interference from forage species was extremely important in regulating *C. nutans* population density, growth rate, and reproductive output of individual plants. The present study has provided evidence, through the use of a comparative approach, that this inhibition has an allelopathic basis and that forage species and cultivars with strong allelopathic effects may have a considerable and largely unrealized potential in the management of problematic weed species such as *C. nutans*.

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MALE SEX PHEROMONE OF A GIANT DANAINE BUTTERFLY, *Idea leuconoe*

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Abstract—Males of a giant danaine butterfly, *Idea leuconoe*, display hairpencils during courtship. The females were visually attracted to and olfactorily arrested by an artificial butterfly model to which male hairpencil extracts were added. The hairpencil extracts contained a complex mixture of volatiles, including pyrrolizidine alkaloid (PA) derivatives (danaidone, viridifloric β-lactone), aromatics (phenol, *p*-cresol, benzoic acid), terpenoids (geranyl methyl thioether, (*E*,*E*)-farnesol), a series of γ-lactones (6-hydroxy-4-undecanolides and its homologs), hydrocarbons [(*Z*)-9-tricosene, etc.], and several compounds with higher molecular weight. A mixture of the major volatiles applied to a butterfly dummy strongly elicited an abdomen-curling acceptance posture in females. Viridifloric β-lactone and danaidone induced significant electroantennogram responses on the female's antennae, suggesting their principal role together with other hairpencil components as a sex pheromone to seduce females. *I. leuconoe* males seem to acquire the precursor for both of the PA fragments from the host plant, *Parsonsia laevigata* (Apocynaceae), during the larval stage; thereby they do not show pharmacophagous behavior towards PA-containing plants during the adult stage. However, males are pharmacophagously attracted to and feed on a number of simple phenolic compounds in a manner similar to other danaine species towards PAs. Wild

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males sequester one of the phagostimulants, (−)-mellein, in the hairpencils in varying quantities. Phenolic compounds incorporated in the hairpencils may act primarily as warning odors linked with the defensive PAs present in the body tissues.

Key Words—Lepidoptera, Danainae, *Idea leuconoe*, hairpencil, pheromone, *Parsonsia laevigata*, pyrrolizidine alkaloid, mellein, defense.

INTRODUCTION

A giant danaine butterfly, *Idea leuconoe* Erichson, is found in coastal habitats from the Malay Peninsula to Java, the Philippines, Taiwan, and the Ryukyu Islands (Okinawa, Japan) (Ackery and Vane-Wright, 1984). The larvae feed exclusively on *Parsonsia laevigata* Alston (Apocynaceae) in the Ryukyus. The host plant contains a series of pyrrolizidine alkaloids (PAs) (Abe and Yamauchi, 1987; Abe et al., 1990, 1991), and the imago stores large quantities of the sequestered alkaloid components in its body tissues (3 mg/insect, as *N*-oxide forms) (Nishida et al., 1991, Kim et al., 1994). Thus, unlike most other danaines, the adult males do not seek PAs.

The abdominal hairpencils of *I. leuconoe* males are composed of a pair of large brushes (approximately 15 mm when fully elongated) and a pair of small brushes (approx. 2 mm as the bundle), each independently housed in its own sheath (Figure 1). The male everts the hairpencils during courtship while hovering above a female, thus disseminating volatile chemicals from the hairpencils. The large bundles of the hairpencils occasionally touch the female's antennae or a part of the body, and then the female accepts him and copulation occurs. The hairpencil components were suggested to play a decisive role as a male sex pheromone, as has been shown in the queen butterfly, *Danaus gilippus* (Pliske and Eisner, 1969).

Upon predatory attacks, the males also display hairpencils, disseminating a phenol-like odor. This suggests a secondary function of the hairpencil volatiles as repellents or as an aposematic odor in conjunction with the PAs sequestered in the body. Although *I. leuconoe* males do not show affinity to PAs, they are strongly attracted to some phenolic substances (Nishida et al., 1990), which implies a pharmacophagous association different from all other danaine species.

We report here on the identification of the hairpencil components and their biological roles as a sex pheromone and possibly as an allomone in this danaine species.

METHODS AND MATERIALS

Instruments. Gas chromatography (GC) -electron impact mass spectra (EI-MS) for the volatile components were obtained with a Hitachi M-80 mass

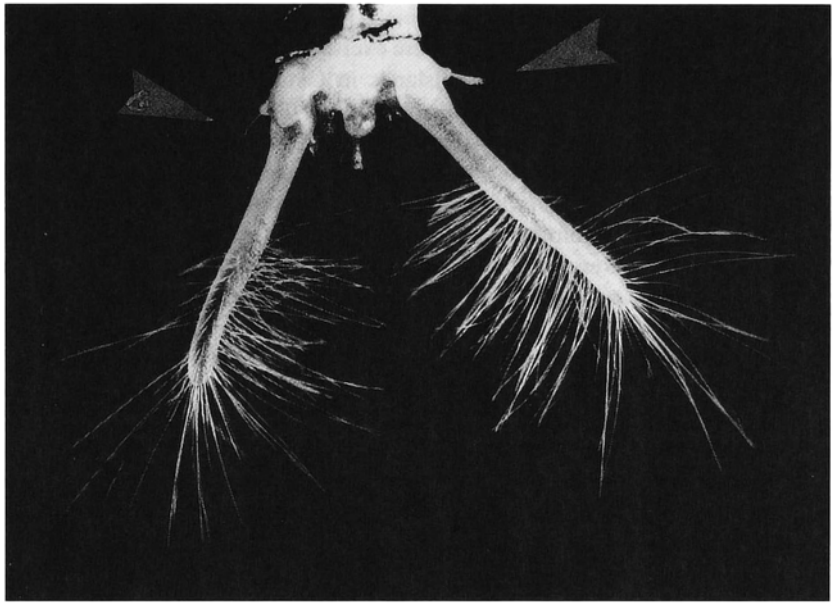


FIG. 1. Extruded hairpencils of *Idea leuconoe* male. A small pair of hairpencils (arrows) are situated on the sheath of a large pair.

spectrometer (70 eV) connected to a GC column (24-m × 0.25-mm fused silica column coated with cross-linked methyl silicone HP-1, 0.33-μm film thickness, helium as carrier gas) programmed from 80°C (approximately 2 min holding) to 240°C at a rate of 10°C/min unless otherwise mentioned. Less volatile substances were analyzed by a GC-MS system (VG 70/250 S mass spectrometer coupled to a Hewlett-Packard HP 5890A gas chromatograph using a CP-Sil-8-CB (30-m × 0.32-mm, 0.25-μm film thickness, Chrompack fused silica column with hydrogen as carrier gas and different temperature programs). High-resolution mass spectra were obtained with the same system. Chemical ionization (CI) -MS measurements were done with isobutane under similar GC conditions using a Shimadzu GC-MS QP1000EX. A chiral GC column (CP-Cyclodextrin B-2,3,6-M-19, Chrompack, 25 m × 0.25 mm, 0.25-μm film thickness, 150°C isothermal, helium) was used for determination of the absolute configuration of mellein. GC-FT-infrared (IR) spectra were obtained with a Shimadzu IRG-8000 spectrometer connected to a capillary column (30 m × 0.53 mm CBJ 17-W30-100, 1.0-μm film thickness, with helium as carrier). The ^{1}H NMR (90 MHz) and ^{13}C NMR (22.5 MHz) spectra were recorded with a Jeol JNM FX-90Q spectrometer with TMS as an internal standard.

Extraction of I. leuconoe *Hairpencil Volatiles.* Hairpencils (large and small pairs of the bundles combined) were excised from 40 males collected from field sites in Ishigaki and Iriomote islands in Okinawa Prefecture at various seasons and were extracted with ethanol (40 ml). The residue was reextracted twice with 4 ml chloroform–ethanol (1 : 1), and the combined extracts were evaporated at 25°C (20 mm Hg) to yield an oily material (53 mg). This extract was partitioned between chloroform (10 ml) and water (5 ml), the water phase extracted again with chloroform, and the combined chloroform layers dried over anhydrous Na_2SO_4. Removal of the solvent by evaporation under reduced pressure gave a yellow oil (11.5 mg), which was chromatographed on a silica gel column (0.5 g, Wako gel C-200) into 18 fractions eluting in sequence with a mixture of increasing concentrations of methyl acetate (%) in hexane (v/v, 5 ml each) as follows: fractions 1 (0%), 2 (1%), 3 (2%), 4 (3%), 5 (4%), 6 (5%), 7 (7%), 8 (9%), 9 (12%), 10 (15%), 11 (20%), 12 (25%), 13 (32%), 14 (40%), 15 (50%), 16 (60%), 17 (72%), 18 (100%).

Isolation of Compounds ***4*** *and* ***11.*** Fractions 9 and 10 were combined and subjected to preparative HPLC (Nucleosil 100-5, 8 mm ID × 300 mm). Fractions were eluted with 20% methyl acetate in hexane at a flow rate of 2 ml/min and monitored with a refractive index detector (Knauer Differential Refractometer). Compounds **4** and **11** were found at retention times (R_t) of 13.0 min and 13.2 min, respectively. Both compounds were purified further using the same HPLC column eluted with 8% methyl acetate + 6% acetone in hexane. Compound **11** was isolated at R_t = 15.1 min (yield, approx. 0.5 mg), and compound **4** was isolated at R_t = 16.6 min (yield, approx. 0.2 mg).

Quantifications of Hairpencil Volatiles. The hairpencil complex was excised from laboratory-reared males that were raised on *Parsonsia laevigata* and fed with sucrose solution during the adult stage and kept in small cages, at 22–25°C and 16 : 8 light–dark photoperiod. The hairpencil complex was excised at 5, 10, 15, 20, and 30 days after emergence, and individually extracted with ethanol (0.5 ml/male). Portions of supernatants of the crude extracts after grinding and ultrasonication were subjected to GC analyses. Excised hairpencils from wild males were processed in a similar manner. GC quantification of volatiles was done on an HP 5790A gas chromatograph with the same HP-1 column and conditions as those employed for the GC-MS analysis. Comparison of the FID intensities with those of the standard samples of known concentrations was performed using an HP 3390A reporting integrator (Hewlett Packard).

Behavioral Bioassay. Virgin females of *I. leuconone* (two to four weeks after adult eclosion) were released in a greenhouse (approx. 50 m^3) where the host plant and some nectar sources (host flowers and sucrose solution) were supplied. A butterfly dummy was made with a sheet of paper (Ricopy PPC paper type 6200, Ricoh) by copying the intact wings of an *I. leuconoe* male with a

copying machine and the dummy glued on a small piece of plastic plate connected to a glass rod; a thin piece of filter paper disk (Advantec Toyo 4A, 21 mm diameter) was attached to the end of the rod where the everted hairpencils would be situated (see Figure 6A below). When the model was moved manually by waving the rod as though the butterfly were flying, the patrolling virgin female usually approached regardless of the presence or absence of the hairpencil chemicals. Within a 1-m range of the model, individual females approaching randomly were scored for touching, landing, and eventual abdomen-curling on the model during a test period of 15 sec.

Feeding Bioassay of Mellein. The pharmacophagous acquisition of mellein was examined using a pure sample of (−)-mellein isolated from a fungus, *Lasiodiplodia theobromae* (provided by Dr. H. Nago) (Matsumoto and Nago, 1994). Laboratory-reared males (25 days old) were allowed to feed on 60 μg of the compound for about 1 hr. Thereafter they were given sucrose solution daily, and the hairpencil complex was removed five days after feeding on (−)-mellein and processed in the same manner as described above for GC quantifications.

Electroantennogram (EAG) Recording. EAG responses were recorded from antennae of 10- to 14-day-old adults of both sexes. An antenna was excised at its base and mounted upward on a plastic plate that was placed in Ringer's solution (Roelofs and Comeau, 1971). An AgCl-coated Ag plate was used as the indifferent electrode. The recording electrode consisted of a glass capillary filled with Ringer's solution (Pt wire inserted) and was brought into contact with the distal end of the excised antenna. This preparation was connected to a DPZ-115 input probe coupled with a DPA-100 amplifier (Dia Medical System), which was interfaced to a NEC PC-9801m microcomputer. EAGs were visualized on a CRT and measured as the maximum amplitude of depolarization elicited by a stimulus. An odor puff of 2 ml was mixed with a humidified airstream blowing continuously over the antenna at a rate of 350 ml/min through 6-mm-ID glass tubing, which terminated 1 cm from the antennal preparation. A test compound dissolved in dichloromethane was adsorbed on a piece of filter paper (5 × 30 mm). After evaporating the solvent at room temperature, the filter paper was placed in a sample cartridge (6 mm ID × 50 mm), and puffed into the airstream. Each chemical was tested at two doses (0.1 and 1.0 μg) on 10–14 male antennae and 10–11 female antennae, each of which originated from a different butterfly. All tests were repeated five times for each antenna. Since the antennal responses decreased in amplitude over time and the interindividual variation in absolute response to a stimulus was considerable, standards (10 μg of 1-hexanol) and blanks were included before and after each test chemical. The responses were averaged and expressed as percentages of the response to the standard.

RESULTS

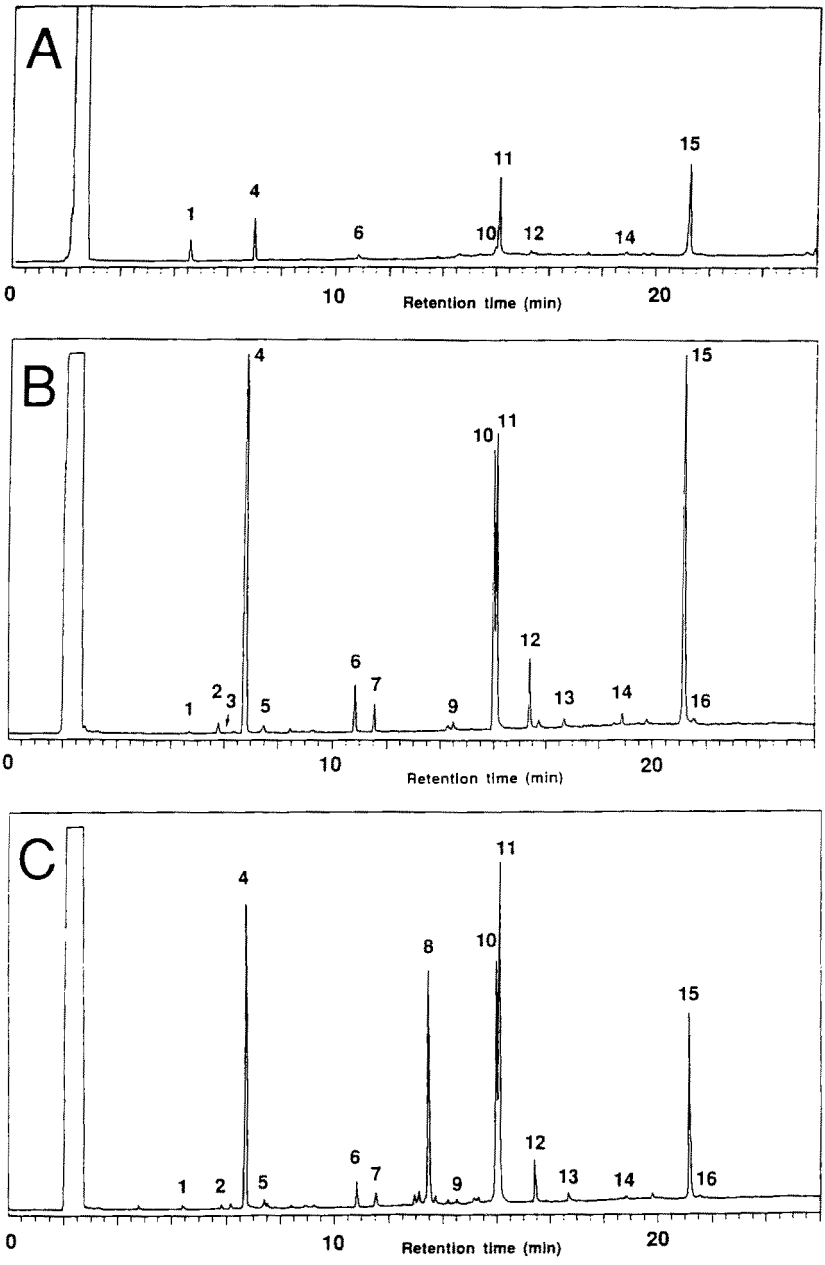
Identification of Hairpencil Volatiles

Small and large bundles of hairpencils excised from sexually mature indoor males of *I. leuconoe* were analyzed separately by means of GC-MS as shown in Figure 2A and 2B. The chromatograms exhibited a series of volatile substances numbered from **1** to **16.** Although the quantities of the volatiles in the small hairpencils were extremely low compared to the larger ones (e.g., less than one thirtieth of compound **15** contained in the small ones), both shared the same compounds in different ratios. Figure 2C shows the hairpencil volatiles from males captured in Iriomote Island (large and small bundles combined). The profile was very similar to that of indoor males, but most of the males contained compound **8,** which was absent in the indoor males. Figure 3 shows mass spectra of the hairpencil components (**2, 4, 6, 7, 8,** and **10**). The hairpencil volatiles appeared to contain a variety of compounds belonging to several different classes. In order to facilitate the structural elucidation of unknown compounds, some of the components were isolated in pure forms from hairpencil extracts of outdoor males by column chromatography and preparative HPLC. Each component was characterized by spectrometric analyses and also by syntheses, if necessary, as described below.

Simple Aromatics (1, 3, 5). Compounds **1** and **3** were found to be responsible for the characteristic strong odor of the hairpencils. Compound **1** (EI-MS: *m/z* 94, 66, 65, 39) and compound **3** (EI-MS: *m/z* 108, 107, 79, 77, 51) were identified as phenol and *p*-cresol, respectively, while compound **5** proved to be benzoic acid (EI-MS: *m/z* 122, 105, 77), by comparing their gas chromatographic and mass spectroscopic data with the authentic samples.

β-Lactones (2 and 4). Compound **4** was isolated by column chromatography (fractions 9 and 10). The EI-MS (Figure 3) showed prominent ions at *m/z* 100, 85, 57 and 43, while the CI-MS exhibited ions at *m/z* (%) 145 (M+H, 40), 127 (54), 117 (100) and 101 (60). The ^{1}H NMR spectrum of compound **4** indicated the presence of an isopropyl group (δ2.27, 1H, septet, J = 6.8 Hz; 1.16, 3H, doublet, J = 7 Hz; 0.98, 3H, doublet, J = 6.6 Hz) and a secondary methyl group (δ1.54, 3H, doublet, J = 6.5 Hz) coupled with a methine proton

FIG. 2. Gas chromatograms of volatiles in the extracts of *Idea leuconoe* male hairpencils. (A) Small pair of hairpencils from indoor males. (B) Large pair of hairpencils from indoor male. (C) Hairpencil complex (large and small pairs combined) of wild males captured at Iriomote Island. Chromatograms were done on a capillary HP-1 column (24 m × 0.25 mm, programmed from 80 to 240°C, 10°C/min) with a total ion monitor (*m/z* 33–330).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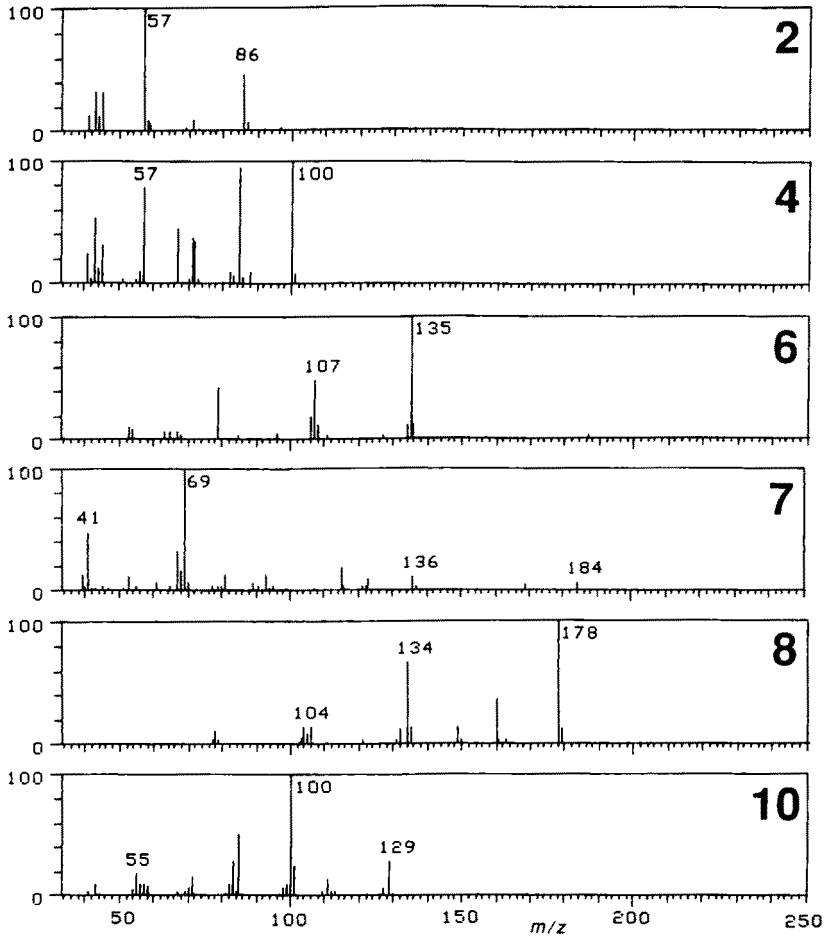

FIG. 3. Mass spectra of compounds **2**, **4**, **6**, **7**, **8**, and **10** (EI-MS, 70 eV).

(δ4.62, 1H, quartet, J = 6.5 Hz), the spectral pattern of which was similar to that of a necic acid fragment, either viridifloric acid or trachelanthic acid (Nishida et al., 1991). These data suggested a β-lactone structure for compound **4.** The FT-IR spectrum of **4** gave a strong absorption at 1850 cm^{-1}, which is characteristic of a carbonyl stretching band of a β-lactone (Figure 4). Conclusive evidence was obtained by an unequivocal synthesis of the corresponding β-lactones both from (−)-viridifloric acid and (+)-trachelanthic acid by cyclization with methansulfonyl chloride (Schulz and Nishida, 1996). The spectral

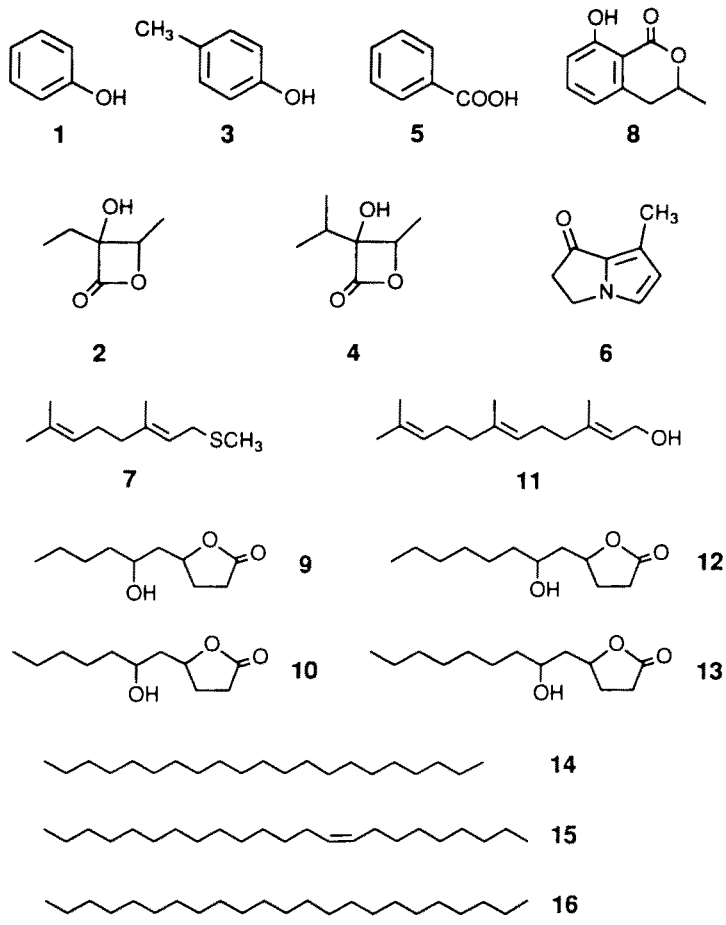

FIG. 4. Hairpencil volatiles of *Idea leuconoe* male. **1:** Phenol, **2:** 2-hydroxy-2-ethyl-3-butanolide, **3:** *p*-cresol, **4:** 2-hydroxy-2-(1-methylethyl)-3-butanolide (viridifloric β-lactone), **5:** benzoic acid, **6:** danaidone, **7:** geranyl methyl thioether, **8:** (−)-mellein, **9:** 6-hydroxy-4-decanolide, **10:** 6-hydroxy-4-undecanolide, **11:** (*E*,*E*)-farnesol **12:** 6-hydroxy-4-dodecanolide, **13:** 6-hydroxy-4-tridecanolide, **14:** heneicosane **15:** (*Z*)-9-tricosene **16:** tricosane.

(EI-MS, ^{1}H-NMR) and chromatographic (GC) data of compound **4** coincided with the β-lactone derived from viridifloric acid. Thus compound **4** was determined as viridifloric β-lactone [(2*RS*,3*RS*)-2-hydroxy-2-(1-methylethyl)-3-butanolide]. Since *I. leuconoe* incorporate (+)-lycopsamine *N*-oxide, which

contains the (−)-viridifloric acid moiety, from its host *P. laevigata*, the β-lactone is assumed to possess the same 2*S*,3*S* configuration.

Compound **2** was deduced to be a nor-derivative of β-lactone **4** (2-hydroxy-2-ethyl-3-butanolide) (Figure 4), because its mass spectrum showed ions at *m/z* 131 (M+H) in the CI-MS and at *m/z* 86 (M-CO_2) in the EI-MS (Figure 3). This was also supported by the evidence that ideamine A *N*-oxide (a nor-analog of lycopsamine *N*-oxide possessing a 2-ethyl-2,3-dihydroxy moiety) is one of the major PAs sequestered by the imago (Nishida et al., 1991).

Danaidone (6). The presence of danaidone (**6**) (found predominantly in column chromatographic fraction 13) was confirmed by its diagnostic GC-MS behavior (Figure 3) in comparison with a synthetic sample.

Terpenoids (7 and 11). The molecular formula of compound **7** was determined to be $C_{11}H_{20}S$ by GC-HRMS (M^+: obs. 184.1286, calc. 184.1289). Compound **7** gave a mass-spectrometric fragmentation pattern with some similarity to geraniol (Figure 3) and characteristic sulfur-containing ions at *m/z* 45, 61 and 115, suggesting a methyl thioether structure of a monoterpene. Geranyl methyl thioether was prepared by reaction of geranyl chloride with sodium thiomethanoate (Schulz and Nishida, 1996). The gas chromatographic and mass spectroscopic data of the thioether completely coincided with that of **7**. Thus, **7** was assigned to be (*E*)-3,7-dimethyl-2,6-octadienyl methyl thioether (Figure 4).

Compound **11** was identified as (*E*,*E*)-farnesol by comparison with an authentic sample (Aldrich Chemical Co.); no other geometrical isomers were present in the hairpencil extracts. MS: *m/z* (%) 222 (4), 204 (1), 136 (16), 93 (27), 81 (42), 69 (100), 41 (18). ^{1}H NMR ($CDCl_3$): δ5.40 (1H, broad triplet, J = 6.5 Hz), 5.10 (2H, multiplet), 4.13 (2H, doublet, J = 6.8 Hz), 2.05 (8H, multiplet), 1.67 (6H, broad singlet), 1.60 (6H, broad singlet).

Mellein (8). This compound was recovered from fractions 6 and 7 of the column chromatography and identified as mellein from its diagnostic GC-MS data (Figure 3) and ^{1}H NMR spectrum. ^{1}H NMR ($CDCl_3$) δ11.02 (1H, singlet), 7.40 (1H, double doublet, J = 8.4, 7.5 Hz), 6.88 (1H, doublet, J = 8.4 Hz), 6.67 (1H, doublet, J = 7.5 Hz), 4.72 (1H, sextet, J = 6.6 Hz), 2.92 (2H, doublet, J = 7.0 Hz), 1.52 (3H, doublet, J = 6.2 Hz). The absolute configuration of **8** was determined to be (−)-*R* by comparing its GC-retention time (R_t = 16.52 min) on a chiral column with optically active samples of mellein [(−)-*R*: R_t = 16.52, (+)-*S*: R_t = 16.68 min].

γ-Lactones (9, 10, 12, 13). The column chromatographic fractions 14 and 15 were found to consist of four homologous compounds, **9, 10, 12,** and **13,** all of which gave a characteristic mass spectrometric fragmentation pattern with three prominent peaks at *m/z* 129 (highest mass fragment), 100 (base peak), and 85 (Figure 3). Molecular weights of compounds **10** and **12** were determined to be 200 and 214, respectively, from their CI mass spectra [**10,** *m/z* (%) 201

(65), 183 (100), 165 (53); **12**, *m/z* (%) 215 (48), 197 (77), 179 (51)]. FT-IR spectra of **10**, **12**, and **13** exhibited strong bands at 1810 cm^{-1}, suggesting the presence of a γ-lactone moiety in these compounds. The ^1H and ^{13}C NMR spectra of fractions 14 and 15 (as a mixture of **9**, **10**, **12**, and **13**) confirmed the presence of a γ-lactone moiety with a hydroxy group on the side chain. The ^1H NMR spectrum ($CDCl_3$) of the homologous mixture gave signals at δ4.80 (1H, multiplet), 3.89 (1H, multiplet), and 0.89 (3H, virtually coupled triplet, J = 5.5 Hz). The highly splitting coupling pattern arising from the lactone ring protons in the range of 1.8–2.7 ppm resembled the pattern observed in that of γ-nonalactone. The ^{13}C NMR spectrum ($CDCl_3$) of the mixture gave 11 major signals arising mainly from compound **10** at 176.93, 78.17, 68.64, 43.29, 38.14, 31.80, 28.88, 28.60, 25.19, 22.64, and 14.03 ppm. The hydroxy group was assigned to be attached to C-6, since the prominent peaks at *m/z* 129 and 100 in the EI-MS (Figure 3) can be explained by cleavage at the side of the carbinol group. Thus, compounds **9**, **10**, **12**, and **13** were characterized as 6-hydroxy-4-decanolide, 6-hydroxy-4-undecanolide, 6-hydroxy-4-dodecanolide, and 6-hydroxy-4-tridecanolide, respectively. This structural assignment was confirmed by synthesis of racemic 6-hydroxy-4-undecanolide. The absolute configuration of the natural compound **12** could be established to be 4*S*,6*S* by stereoselective synthesis and chiral gas chromatography (Schulz and Nishida, 1996).

*Hydrocarbons (**14**, **15**, **16**).* Compounds **14**, **15**, and **16** were eluted in fraction 1 from column chromatography. Compounds **14** and **16** were identified as heneicosane and tricosane, respectively. Compound **15** [^{13}C NMR ($CDCl_3$) 129.91, 31.91, 29.69, 29.36, 27.25, 25.62, 24.97, 22.70, 14.09] was identified as (*Z*)-9-tricosene by dimethyldisulfide (DMDS) derivatization (Francis and Veland, 1981) of the hydrocarbon mixture (fraction 1) and direct comparison of the adduct with those of authentic samples of (*E*)- and (*Z*)-9-tricosene. DMDS derivative of **15**: MS *m/z* (%): 416 (M^+, 8), 243 ($C_{11}H_{28}SCH_3$, 89), 173 ($C_9H_{18}SCH_3$, 100). Besides these compounds, small amounts of the following straight-chain hydrocarbons were also detected: nonadecane, nonadecene, 7- and 9-heneicosene, 7-, 8-, and 9-docosene, tricosadiene, tetracosane, 7- and 9-tetracosene, pentacosane, 7- and 9-pentacosene, hexacosane, and heptacosane.

Higher Boiling-Point Compounds. Besides the volatile compounds eluting before tricosane on our GC column, several other lipidic components could be identified by their characteristic mass spectra and comparison with selected synthetic samples. They consisted mainly of straight-chain ketones, secondary alcohols, 2,5-dialkyltetrahydrofurans (Schulz et al., 1993), and steroids. The ketones form regioisomeric mixtures of C_{21}, C_{23}, C_{24}, and C_{25} compounds, for example 2-, 3-, 4-, 5-, 6-, 7-, 9-, or 10-pentacosanone. Corresponding secondary alcohols as unsaturated compounds were also present. The tetrahydrofurans occur as *trans* and *cis* isomers with chain lengths between C_{27} and C_{33}. Most prominent was 2-hexadecyl-5-nonyltetrahydrofuran, accompanied by 14 other tetrahydrofurans.

Cholesterol, ergosterol, sitosterol, and α-tocopherol were also detected in small quantities. Full details of the identified lipids will be given elsewhere (Schulz and Nishida, 1996). However, it has not been confirmed whether these compounds originate exclusively from the hairpencil organs, or whether they are also present on the cuticle and/or blood of the butterflies.

Figure 4 summarizes the structures of the volatile components (**1–16**) characterized from hairpencil extracts of *I. leuconoe* males.

Changes in Contents of Hairpencil Volatiles during Aging

The contents of hairpencil volatiles of indoor-raised *I. leuconoe* males were roughly quantified by GC. Figure 5 shows the mean contents of compounds **4, 6, 10,** and **15** from the hairpencil complex (small and large bundles combined) by age (5, 10, 15, 20, and 30 days after adult emergence). The hairpencils from 5-day-old males contained volatiles in trace amounts, although the extruded hairpencils possessed a faint phenol-like odor. The mean contents (±SE) of the major volatiles at the 20th day were as follows: **4:** 6.3 ± 0.7, **6:** 1.0 ± 0.3, **7:** 1.0 ± 0.1, **10:** 14.6 ± 2.0, **11:** 6.8 ± 1.2, **15:** 23.1 ± 3.4 μg/male (N = 5). The amounts of other hairpencil volatiles also increased more or less proportionally with age, although the quantities varied individually.

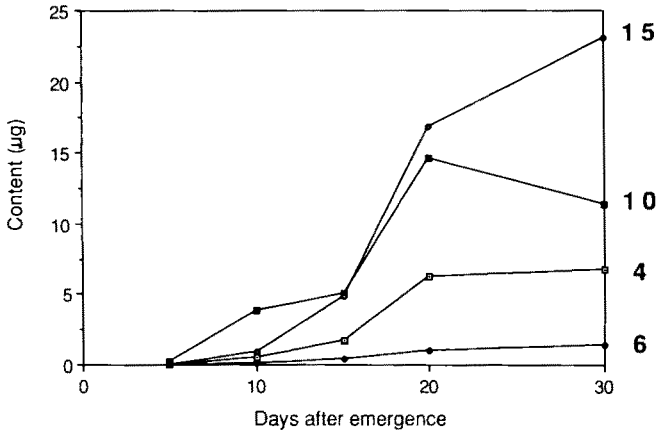

FIG. 5. Mean contents of viridifloric β-lactone (**4**), danaidone (**6**), 6-hydroxy-4-undecanolide (**10**), and (*Z*)-9-tricosene (**15**) in the hairpencil complex of *Idea leuconoe* males by age (N = 5).

TABLE 1. MEAN CONTENTS OF MELLEIN IN HAIRPENCIL COMPLEX OF LABORATORY MALES OF *Idea leuconoe* UNFED AND FED WITH MELLEIN AND OF OUTDOOR MALES

Males analyzed	Mellein content (± SE μg/male)	*N*
Unfed[a]	0	5
Fed with mellein[a]	8.9 ± 2.2	6
Wild (May 1990)[b]	4.2 ± 1.2	19
Wild (November 1991)[b]	4.3 ± 1.8	6

[a]Indoor males (30 days old, five days after treatment with mellein).
[b]Males captured at field sites in Iriomote Island.

Incorporation of Mellein (8) in Hairpencils

Males of *I. leuconoe* showed strong affinity to one of the hairpencil components, mellein, and ingested the compound by dissolving it with saliva. Most of the males quickly extended their proboscis as soon as the sample was brought near them, and they continued to feed for more than 30 min. Mellein was incorporated in the hairpencil complex as shown in Table 1. GC profiles of the hairpencil extracts in mellein-fed males were very similar to those in some of the males captured at field sites, although the quantities of the compounds varied individually. Mellein was scantily detected (0–0.02 μg/male) in the small pair of hairpencils, while a larger portion was accumulated in the large pair, sometimes over 15 μg/male.

Pheromonal Effect of Hairpencil Volatiles towards Females

In the mating sequence of *I. leuconoe*, males usually chase flying females and repeatedly display hairpencils in the female's vicinity, most frequently hovering above the female's head. In response to the hairpencil display, a receptive female lands on a nearby plant leaf, closes her wings, and curls her abdomen downward, which seems to be a typical acceptance posture of the female. Copulation takes place immediately afterwards.

Some of the females displayed the same abdomen-curling behavior when exposed to excised hairpencils or a piece of filter paper impregnated with crude extracts of hairpencils from mature males (0.1 male equivalent/paper disk). If the hairpencil extract was supplied together with a paper model of the butterfly, the females responded more effectively to the volatiles, particularly when the model was "fluttering," as shown in Figure 6A. Moreover, the receptive virgin

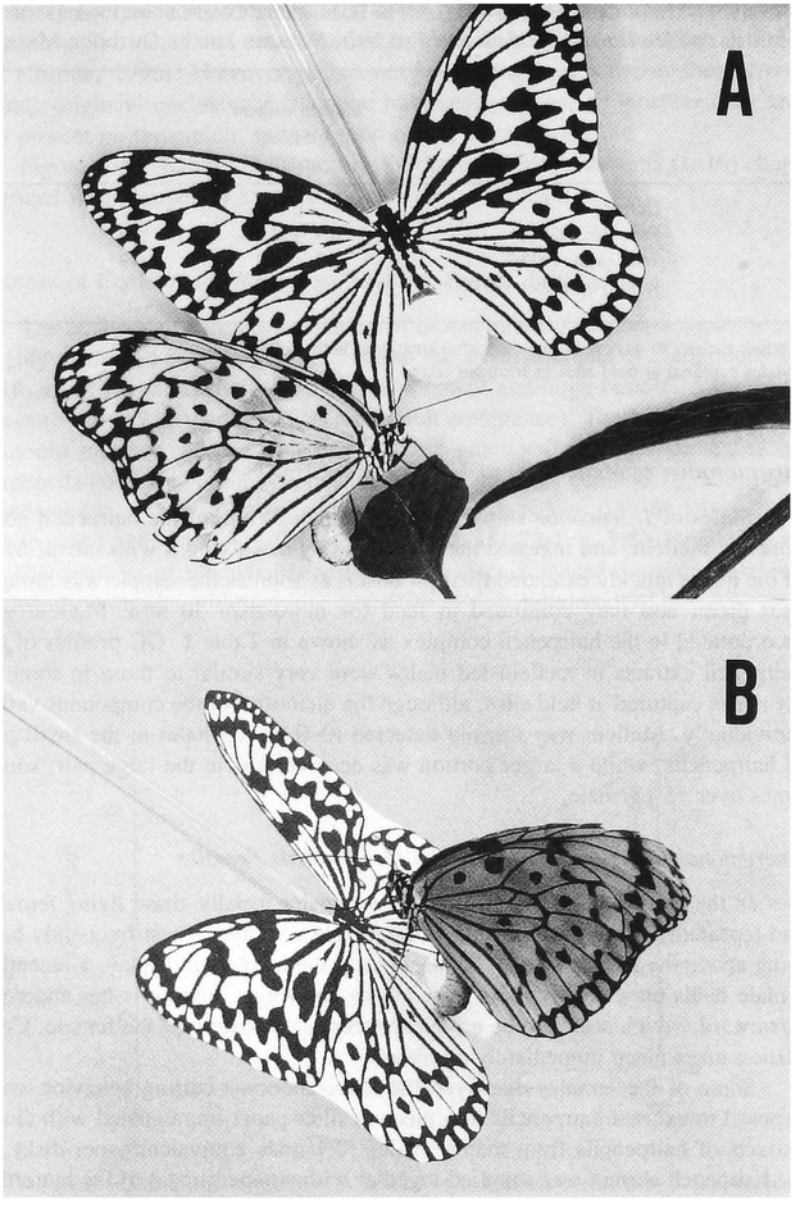

FIG. 6. (A) *Idea leuconoe* female in acceptance posture, visually and chemically stimulated by a paper model of a male with a paper disk impregnated with the hairpencil extract. The female is strongly arrested, exposing the tip of her abdomen from the

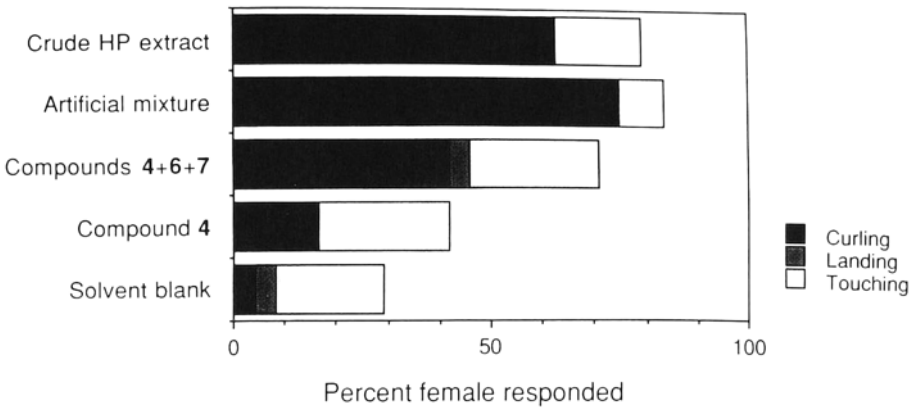

FIG. 7. Behavioral responses of *Idea leuconoe* females to hairpencil (HP) extract and blends of compounds isolated from males. Crude HP extract: 0.1 male equivalent/disk. Artificial mixture (compounds **1** + **3** + **4** + **5** + **6** + **7** + **9** + **10** + **11** + **12** + **13** + **15**): approx. 0.1 male equivalent/disk. Compounds 4 + **6** + **7**: 5.0:0.5:0.5 μg dose. Compound **4:** 5.0 μg dose. Female approaches to a butterfly model plus chemical samples were scored for touching the model (light gray), landing on the model (dark gray), and abdomen-curling behavior (black) (N = 24 for each treatment).

female (2–4 weeks old) actively chased the fluttering model, and landed on the model wings with abdomen-curling posture, sometimes firmly pushing the tip of her abdomen toward the sample paper disk (Figure 6B).

Figure 7 shows behavioral responses of virgin females to the crude hairpencil extract and hairpencil components tested with the fluttering-paper model by the method shown in Figure 6B. Crude HP extract at a dose of 0.1 male equivalent/dummy was sufficient to attract and arrest females on the dummy. An artificial mixture of compounds **1, 3–13,** and **15** induced abdomen-curling response to the same level as that of the crude extract (response not significantly different from the crude extract, chi-square 2 × 2 test of independence with Yates' correction, $P > 0.05$). A mixture of **4, 6,** and **7** also exhibited activity to some extent (response significantly different from solvent blank, $P < 0.01$), but the abdomen-curling activity of compound **4** alone was not significantly different from the solvent blank at $P = 0.05$.

hindwings. (B) The female landed on the paper model plus paper disk treated with an artificial blend of hairpencil components (**1** + **3** + **4** + **5** + **6** + **7** + **9** + **10** + **11** + **12** + **13** + **15**), curling her abdomen.

EAG Responses to Hairpencil Components

EAG responses of *I. leuconoe* males and females to some of the hairpencil volatiles are shown in Figure 8 [a mixture of hydroxy-γ-lactones (**9** + **10** + **12** + **13**) used for the test was obtained from chromatographic fractions 14 and 15]. Average EAG amplitudes differed by a factor of 3 between the least and most stimulating compounds. Although stronger responses were evoked at a higher dosage (1.0 μg), the dose-response relation appeared not to be strong. However, relative responses of female and male antennae to viridifloric β-lactone (53.3–83.6%) and danaidone (54.8–90.5%) were higher than those to the other hairpencil components (25.8–55.6%), and female-to-male ratios of

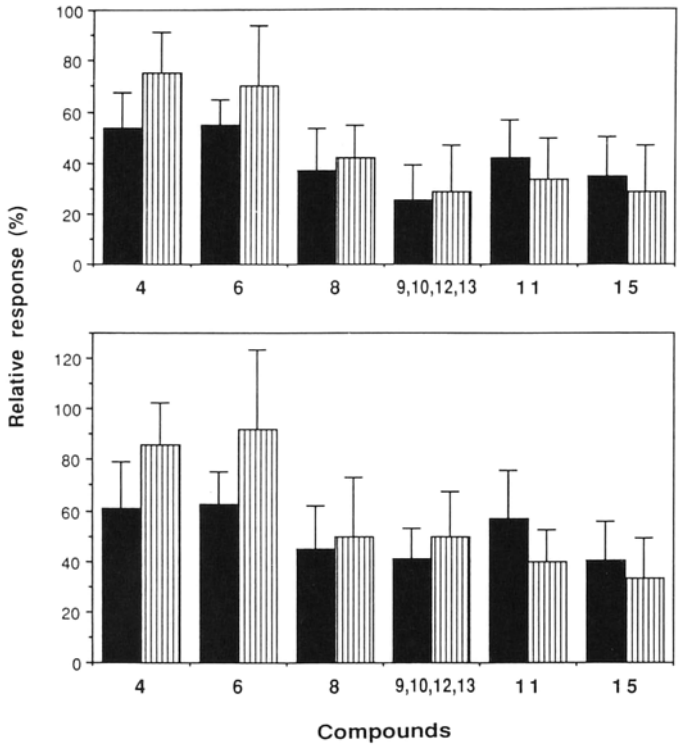

FIG. 8. Relative EAG responses of males (black) and females (striped) to hairpencil components: viridifloric β-lactone (**4**), danaidone (**6**), mellein (**8**), a mixture of 6-hydroxy-4-alkanolides (**9** + **10** + **12** + **13**), (*E*,*E*)-farnesol (**11**), and (*Z*)-9-tricosene (**15**). Relative values to the response for standard 1-hexanol at the same dosages are given. Upper: 0.1-μg dose, lower: 1.0-μg dose.

responses to viridifloric β-lactone (1.4) and danaidone (1.3–1.5) were somewhat larger than to the others (0.7–1.2).

DISCUSSION

Similar to other danaine species (Brower et al., 1965; Pliske and Eisner, 1968; Ackery and Vane-Wright, 1984), the courtship behavior of *Idea leuconoe* consists of a complex behavioral sequence with some variations. In a typical interaction, a male displays hairpencils in aerial pursuit of a female. A receptive female alights on available herbage nearby. The male hovers above her and repeatedly extrudes and retracts his hairpencils in front of her. The female assumes a rigid posture with her wings closed and the abdomen curved downward below the hind wings. This posture, which is assumed to be an acceptance response, evidently facilitates copulation. The male alights laterally and eventually copulates.

It has been shown here that the hairpencil volatiles of *I. leuconoe* males elicit a typical acceptance posture in the females when supplied together with the butterfly dummy. The male hairpencil pheromone is composed of a complex mixture. A visual cue as well as the olfactory signal appeared to be involved in induction of female acceptance. Preliminary experiments showed that an air current together with the crude hairpencil extract (0.1 male equivalent on a piece of filter paper per 5-ml air puff) induced a proboscis-extending response in caged virgin females, sometimes accompanied by the abdomen-curling response even without visual cues. During actual courtship, some of the females occasionally uncoil their proboscis halfway in response to a male's hairpencil display prior to abdomen-curling; the same behavior can also be seen toward the model scented with hairpencil extract. The proboscis-extending behavior may be associated with the aphrodisiac response of the female.

After an aerial pursuit by a male, a young virgin female (e.g., one week after emergence) lands on herbage and either passively accepts the hairpencilling male for mating or rejects him by an evasive posture with her wings open. Such young females seldom chased males under greenhouse conditions. However, 2-week-old or older virgin females positively pursued males. Then the females quickly landed on neighboring plant leaves and curled their abdomens, apparently inviting a male. The landing and abdomen-curling behavior on the butterfly dummy looked as though it was a positive copulatory attempt by the female, but this behavior may be a mere arrested reaction when no nearby herbage is available within 1 m from the dummy.

I. leuconoe adults survive for several months (three to five months under laboratory condition at 25 ± 3°C). According to indoor observations, the day of the first mating occurred a few weeks after adult emergence both in males

and females, and most of the males and females mated several times during their lives. The date of the first mating and the mating frequency varied greatly by individual [the mean first mating was 45 days in males (N = 139), and 27 days in females (N = 129) at Kashihara City Insectarium (August 1993–July 1994) (H. Morishima et al., personal communication). The behavioral change in females and relatively slow production of the hairpencil volatiles in males (Figure 5) appeared to be more or less consistent with their sexual maturation.

The hairpencil volatiles of *I. leuconoe* males consist of a complex mixture of several classes of compounds, including simple aromatics (**1, 3, 5**), two β-lactones (**2, 4**), danaidone (**6**), acyclic mono- and sesquiterpenes (**7, 11**), a series of new hydroxy-γ-lactones (**9, 10, 12, 13**), and several straight-chain aliphatic compounds (**14, 15, 16** and higher boiling-point components). Although the contribution of each individual component has not been determined, a blend of the compounds was found to be responsible for seducing females.

Danaidone acts as an aphrodisiac pheromone for *Danaus gilippus* females and is transferred to the female's antennae by means of cuticular particles (pheromone transfer particles) through direct contact with the hairpencils. The particles are glued to the antennae by a terpenoid diol (Pliske and Eisner, 1969). *I. leuconoe* males, which do not appear to possess pheromone transfer particles, usually hover and evert hairpencils above the females, and brush them against a female's antennae and head. The female responded positively even without direct contact to the crude hairpencil extracts. Thus, the hairpencil components are transmitted primarily through air. Nevertheless, the intensity or duration of the pheromonal signal may be enhanced by applying a solution of the pheromone components directly onto the antennae of the female. It seems likely that the high boiling-point ketones, alcohols, and hydrocarbons may function as such a solvent.

Danaidone appears to be one of the important factors of the pheromone blend in *I. leuconoe*. This compound has been identified from a number of danaine species, including the genera *Danaus*, *Amauris*, and *Parantica* (Meinwald et al., 1966, 1969; Edgar and Culvenor, 1975; Edgar, 1982; Petty et al. 1977; Komae et al., 1982; Schulz et al., 1988, 1993; Francke et al., 1989). Precursors for danaidone are acquired by adults while pharmacophagously feeding on PA-containing plants in most of those danaine species (Edgar et al. 1973; Schneider et al., 1975). In contrast, *I. leuconoe* obtains a series of PAs from its own host during the larval stage (Nishida et al., 1991). Thus, the males are likely to convert a portion of the alkaloids to danaidone without pharmacophagous foraging during the adult stage. Interestingly, Abe and Yamauchi (1987) isolated a (−)-viridifloric ester of 1-hydroxymethyl-5,6-dihydropyrrolizin-7-one (parsonine) from stems of the host *P. laevigata*, the necine moiety of which structurally resembles danaidone (1-methyl-5,6-dihydropyrrolizin-7-one). However, the actual precursor(s) of danaidone in *I. leuconone* are not known.

β-Lactones **2** and **4** seem to originate from the other skeletal portion of PA moieties, the necic acids. They are cyclization products of norviridifloric (2-ethyl-2,3-hydroxybutanoic) and viridifloric acids, which correspond to the esterifying acid moieties of ideamine A and lycopsamine, respectively, major PA components sequestered by *I. leuconoe* (Nishida et al., 1991). The β-lactone ring is unique as a sex pheromone. The related ithomiine butterflies use a γ-lactone, ithomiolide A, as pheromone, which is also derived from viridifloric acid (Edgar et al., 1976; Schulz, 1992). β-Lactone **4** and danaidone gave potent EAG responses, in which the antennal signals in females were significantly higher than those in males, providing additional support for the essential contribution of these PA fragments to the pheromone system studied.

Geranyl methyl thioether (**7**) is also a novel compound, although several oxygenated monoterpenic compounds have been characterized from hairpencils of danaine species (Schulz et al., 1988, 1993; Francke et al., 1989). The sulfur-containing monoterpene seems to be a rare example as a component of glandular secretions in insects. The sesquiterpene (*E,E*)-farnesol (**11**) has been identified from some danaine species in the genus *Amauris* (Schulz et al., 1993). A bis-homomonoterpene diol, (*E,E*)-3,7-dimethyldeca-2,6-dien-1,10-diol from the hairpencil of *D. gilippus* acts as a glue for the pheromone transfer particles (Meinwald et al., 1969). (*E,E*)-Farnesol may function as an olfactory cue, since the distinct floral fragrance of hairpencil extracts after evaporation of phenols is mainly due to this compound. It is noted that a relatively higher proportion of **11** was present in the small pair of the hairpencils (Figure 2A). The only other compound described so far from another *Idea* species, *I. iasonia*, is the cyclic bisnorsesquiterpene dihydroactinidiolide (Francke et al., 1989), which is absent in the species investigated by us.

Among a series of 6-hydroxy-γ-lactones (**9, 10, 12, 13**) characterized from *I. leuconoe* hairpencils, the C_{11} analog **10** was found to be the most abundant. These compounds are new natural products and may be biosynthesized by the insects.

Several long-chain hydrocarbons (**14, 15, 16,** and others) were present in the hairpencil extract, in which (*Z*)-9-tricosene (**15**) was the major component. They were accompanied by several other less volatile aliphatic compounds, including a series of alkanones, secondary alcohols, and 2,5-dialkylated tetrahydrofuran derivatives. Considering their abundance in the hairpencils, these compounds may act as solvents to regulate vaporization of highly volatile pheromone components in the hairpencil organs. In addition they function to transmit a set of the volatile pheromone onto the female's antennae through direct contact during brushing as discussed above. The oxygenated molecules may be needed to enhance the solubility of polar components.

Although hairpencil components are essential for induction of female acceptance, males deprived of hairpencil organs (large and small bundles both)

can occasionally mate under insectary conditions. Interestingly, such hairpencil-ectomized males were persistently chased by normal males, and sometimes normal males displayed hairpencils to the dissected males (more than 20 cases of homosexual hairpencilling behavior were observed exclusively from the normal males to the hairpencil-ectomized males) (Nishida, unpublished data). This suggests a secondary pheromonal function of hairpencil components for male–male recognition. The double hairpencil structure is a characteristic of the genus *Idea* (Ackery and Vane-Wright, 1984). The small pair of the hairpencil organs may be involved in the male–male interaction, although it is not currently possible to be sure whether they actually display the small hairpencils during homosexual rejection. The behavioral effect of the hairpencil volatiles toward males is now under investigation.

Males of *I. leuconoe* quickly evert their hairpencils to full length as soon as they are captured in the field, disseminating a characteristic smell of phenol (**1**) and *p*-cresol (**3**). Since the butterfly contains considerable amounts of defensive PAs, these odoriferous compounds seem to act as a warning signal in conjunction with the aposematically yellow-colored hairpencils. The male twists the abdomen to stroke the long-forked hairpencil bundles toward the objects. Simple phenolics have been identified from defensive gland secretions in various arthropods, and the initial phenolic note has been suggested to serve as a highly distinctive early-warning system (Blum, 1981). Phenol and *p*-cresol tested alone did not affect the behavior of *I. leuconoe* females, and their role in the pheromone system remains unknown.

Besides the endogenous volatiles, wild males store an additional component, mellein (**8**), in varying quantities in the hairpencils. The male shows a strong affinity to mellein and selectively accumulates the intact compound within their hairpencil organ (predominantly in the large pairs). A high proportion of the male population in Iriomote Island contained substantial quantities of mellein (over 70% of males examined in May 1990 and November 1991; see Table 1), which implied an ecological role of this compound in nature. The indoor males can mate without access to mellein, and our preliminary bioassay did not give any behavioral evidence of mellein in the courtship sequence.

Mellein is a known fungal metabolite, especially in endophytic species (Yubata and Sumiki, 1933; Arakawa et al., 1969; Aldridge et al., 1971; Matsumoto and Nago, 1994). Nago and Matsumoto (1994) isolated a mellein-producing fungus, *Lasiodiplodia theobromae*, from the body surface of the *I. leuconoe* males captured in Iriomote Island. They suggested a possible ecological significance of mellein in a fungus–butterfly association, in which *I. leuconoe* males potentially act as vectors to transmit fungus spores. So far, no other source for mellein in the natural habitat is known. Mellein is also one of the male hairpencil pheromone components of the Oriental fruit moth, *Grapholitha molesta* (Baker et al., 1981; Nishida et al., 1982), and mellein apparently func-

tioned as a male–male pheromone to repel other males (Nishida et al., 1985). Mellein was also shown to be the male wing gland pheromone of the bumblebee wax moth, *Aphomia sociella*. A mellein-producing fungus, *Aspergillus ochraceus*, in the host nests was suggested as a source (Kunesch and Zagatti, 1987). Mellein has also been found in the secretions of many ant species (Brand et al., 1973; Attygalle et al., 1989; Lloyd et al., 1989; Bestmann et al., 1992).

In addition to mellein a number of other phenolic substances act as potent attractants as well as phagostimulants for *I. leuconoe* males. Males are strongly attracted by certain kinds of hair pomades and cosmetics in an insectary, and methyl *p*-hyroxybenzoate (a preservative) was characterized as the active principle (Nishida et al., 1990). Methyl salicylate was another potent attractant, while the isomer, methyl *m*-hydoxybenzoate, was inactive (Nishida et al., 1990). 2-Hydroxy-4-methoxyacetophenone (paeonol) in the plant *Exacum affine* was also reported as an *I. leuconoe* male attractant (Matsumoto, 1994). Not only these exogenous carbonylated phenols but also endogenous phenols **1** and **3** elicit the feeding response in males (Nishida, unpublished). The males that ingested **1** and **3** accumulated additional quantities of the compounds in the hairpencils. Methyl salicylate, *p*-nitrophenol, *o*-cresol, thiophenol, and an isomer of chloromethylphenols were also detected in hairpencil extracts from a number of males captured in the field (Nishida, unpublished data), although their outdoor sources are unknown.

Endogenous phenol and *p*-cresol seem to act primarily as a warning sign against predators. The pharmacophagous behavior of the males towards a variety of phenolic substances and sequestration of those miscellaneous compounds in the hairpencils are very likely to be associated with the defense mechanism of this poisonous butterfly. Since phenols **1** and **3** are highly volatile and the quantities in the hairpencils are always extremely low, these compounds may quickly evaporate during continuous hairpencilling at courtship. The males could intensify the aposematic odor of hairpencils by acquiring additional amounts of volatile phenols by pharmacophagous foraging from certain sources. Mellein may be one such substance, although its sensory activity to predators has not been examined. A methoxy derivative of mellein (8-hydroxy-6-methoxy-3-methyl-3,4-dihydroisocoumarin) is a bitter principle of deteriorating carrots (Sondheimer, 1957). (−)-Mellein tastes bitter as well (Nishida, unpublished data). It might act as a gustatory cue to deter predators.

Male *Idea* butterflies never seek PAs, probably because they already possess substantial quantities from their larval host, *Parsonsia laevigata*. Portions of sequestered PAs seem to be processed into the necine base and necic acid fragments by males, which utilize them as sex pheromones. Nevertheless, the males show pharmacophagous activity towards phenolics and accumulate the compounds in the hairpencils, while most other danaine species exhibit pharmacophagy towards PAs. Although the overall meaning of this behavior by *I.*

leuconoe is not fully understood, the two different types of pharmacophagy may originate in similar adaptive mechanisms in the danaine species (Edgar et al., 1974; Boppré, 1978, 1990).

Among the 12 species of the genus *Idea*, at least two, including *I. leuconoe*, feed on *Parsonsia*, a genus known to contain PAs (Edgar and Culvenor, 1975; Abe et al., 1990, 1991), while others are recorded either from other Apocynaceae or from Asclepiadaceae (Ackery and Vane-Wright, 1984). Several other danaine and ithomiine species also utilize Parsonsieae as the larval host (Edgar, 1984); a primitive ithomiine butterfly, *Tithorea harmonia*, has been shown to incorporate PAs from the Parsonsieae plant, *Prestonia acutifolia*, during the larval stage (Trigo and Brown, 1990). In addition to *Parsonsia*, two asclepiad plants, *Cynanchum formosanum* and *Tylophora hispida*, were recorded as the larval food plant of *I. leuconoe* (Ackery and Vane-Wright, 1984); the presence of PAs in these plants is not known. It has been suggested that the ancestral food plants of the Danainae contained PAs, and the insects retained the ability to acquire PAs through pharmacophagy even after they shifted to other plants (Edgar et al., 1974; Edgar, 1984). It is of great interest to study further the life history of the primitive genus *Idea*, because it might provide a clue for understanding the evolution of pharmacopagous behavior in the subfamily Danainae.

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EFFECTS OF BILBERRY (*Vaccinium myrtillus* L.) LITTER ON SEED GERMINATION AND EARLY SEEDLING GROWTH OF FOUR BOREAL TREE SPECIES

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Abstract—Laboratory and greenhouse bioassays were used to test for inhibitory effects of senescent and decomposed leaves and aqueous extract from bilberry (*Vaccinium myrtillus* L.) against seed germination and seedling growth of aspen (*Populus tremula* L.), birch (*Betula pendula* Roth.), Scots pine (*Pinus sylvestris* L.), and Norway spruce [*Picea abies* (L.) Karst.]. Aqueous extracts from bilberry leaves were inhibitory to aspen seed germination and seedling growth and also induced root damage and growth abnormalities. Addition of activated carbon removed the inhibitory effects of extracts. Senescent leaves reduced pine and spruce seed germination, but rinsing of seeds reversed this inhibition. Senescent leaves were more inhibitory than decomposed leaf litter, suggesting that the inhibitory compounds in bilberry leaves are relatively soluble and released at early stages during decomposition. Spruce was generally less negatively affected by litter and aqueous extracts than the other tested species. This study indicates that chemical effects of bilberry litter have the potential to inhibit tree seedling recruitment, but these effects were not consistently strong. Phytotoxicity is unlikely to be of critical importance in determining success for spruce seedling establishment.

Key Words—*Vaccinium myrtillus*, regeneration failure, seed germination, seedling establishment, activated carbon, germination inhibitors, phenolic compounds, *Populus tremula*, *Pinus sylvestris*, *Picea abies*, *Betula pendula*.

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INTRODUCTION

Bilberry (*Vaccinium myrtillus* L.) is a dominant component of ground vegetation in Eurasian boreal forest biomes (Walter and Breckle, 1989; Havas and Kubin, 1983). When found in dense clonal monospecific stands, bilberry can interfere negatively with tree recruitment, and these effects can be severe (Sarvas, 1950; Sirén, 1955; Pellissier, 1994). Several possible explanations for failure in seed germination and seedling establishment have been proposed, including resource competition, chemical interference, and humus accumulation (Tirén, 1949; Lehto, 1969; André et al., 1987; Pellisier, 1993, 1994; Kuuluvainen, 1994). Seedlings of deciduous and coniferous trees are seldom observed in monospecific bilberry sites (Arnborg, 1943; Sirén, 1955), and it is therefore reasonable to expect that litter interferes with tree establishment. The large amounts of senescent and decomposing leaves of bilberry found in dense stands could potentially interfere with seed germination and growth of tree seedlings through both physical and chemical interactions. The aim of our study was to test this hypothesis by using senescent and decomposed bilberry leaf-litter in a series of germination and root growth bioassays with pioneer and late successional boreal trees as target species.

METHODS AND MATERIALS

Collection of Senescent Bilberry Leaves. Bilberry leaves were sampled in October 1990 during senescence while they were still attached to shoots. The site used for litter collection was a late successional mixed pine–spruce forest of *Vaccinium myrtillus* type (*sensu* Arnborg, 1990), near Umeå in northern Sweden (63°50′N, 20°22′E). The leaves were air-dried on plastic trays at 20°C until no further weight loss was recorded and then stored in a deep freezer (−18°C) until the experiment started.

Seed Source. Seeds of the following four tree species were used in bioassays: aspen (*Populus tremula* L.) seeds from a natural stand (Ålidhem 63°50′N) with a viability of 96%; Scots pine (*Pinus sylvestris* L.) half-sib plantation seeds (Skaholma 66°90′N), with a viability of 96%; Norway spruce [*Picea abies* (L.) Karst.] half-sib plantation seeds (Lillpite 65°00′N), with a viability of 98%; and birch (*Betula pendula* Roth.) seeds from a natural stand (Mattismyran 66°03′N) with a viability of 42%.

Bioassays with Aqueous Extracts. This experiment was performed to test the effect of aqueous extracts of senescent bilberry leaves on seed germination and radicle growth. Extracts were prepared by soaking 12.5 g of dried leaves in 100 ml distilled water with slow continual stirring for 48 hr at 20°C. The solution was then passed through a filter paper (Munktell No. 3) and the extract

was frozen and stored at −18°C until used. This represents the 12.5% extract, and from this, extracts of 10%, 7.5%, 5.0% and 2.5% were prepared. Two milliliters of each water extract were added to 50-mm-diameter Petri dishes containing one sheet of filter paper (Munktell No. 3) and 50 seeds of either aspen, Scots pine, or Norway spruce. An additional treatment was set up in which 0.5 g of finely powdered activated carbon (Labasco) was added to Petri dishes with 2 ml of 10% extract (and containing 50 seeds of aspen). The carbon was expected to detoxify the extract. Distilled water was used as a control. All treatments were replicated five times. The total number of germinated seeds was counted daily for all species until there was no further germination. Aspen root necroses and abnormal seedlings (i.e., those growing in a prostrate manner) were recorded seven days after sowing. Root length was measured for 10 randomly chosen seedlings per Petri dish at the end of the experiment.

Measurements of pH and osmotic effects were made to eliminate the possibility that these might influence observed trends. The osmotic pressure of the 10% bilberry water extract, measured using a vapor pressure osmometer (Wescor 5500), was 84 mmol/kg, while the pH of the extract was 4.3. Germination tests with polyethylene glycol showed that the lowest molality required to cause negative effects on germination of aspen, Scots pine, and Norway spruce seeds was 154 mmol/kg, 146 mmol/kg, and 130 mmol/kg, respectively, which means that the osmotic potential of our solutions was too low to cause inhibition of our test species.

Bioassays with Senescent Bilberry Leaves. An experiment was set up to test for possible inhibitory effects of bilberry leaves against germination of aspen, Norway spruce, Scots pine, and birch. To each of twenty-seven 50-mm-diameter Petri dishes, 0.5 g of air-dried leaves and 8 ml of distilled water were added, and this was left to incubate for 48 hr at 20°C. To five of the Petri dishes, later to be sown with aspen seeds, 0.5 g of finely powdered activated carbon (Labasco) were added after 44 hr. The Petri dishes were sealed with Parafilm during the incubation. Petri dishes with 10 filter-papers and 8 ml distilled water were used as controls. After incubation one sheet of filter paper (Munktell No. 3) was placed on top of the material used in the different treatments and 50 seeds of aspen, Norway spruce, Scots pine, or birch were sown. Five replicates were used for aspen and birch, and six replicates for Scots pine and Norway spruce. The test was run in a greenhouse with a temperature of 20°C and a day–night light ratio of 17:7. Seed germination was measured daily, 7 days for aspen and 21 days for Norway spruce, Scots pine, and birch.

To investigate whether Scots pine, Norway spruce, and birch seeds that had not germinated at the end of the test were nonviable or only quiescent, a water cleaning treatment was performed. The seeds were moved to new Petri dishes containing 20 ml distilled water, and after 3 hr they were rinsed in running tap water for 15 sec. The seeds were then placed in a clean Petri dishes, each

containing one filter paper and 2 ml distilled water. The Petri dishes were placed in the greenhouse, and total seed germination was measured after 17 days.

Bioassays with Senescent and Decomposed Bilberry Leaves. An experiment was set up to compare rooting ability and root growth of pregerminated Norway spruce and Scots pine seeds in senescent and decomposed bilberry leaves. Decomposed bilberry leaves were prepared by placing 21 g air-dried leaves in nylon-net litterbags on the ground under snow in bilberry-dominated vegetation in January 1991. In May the same year, the litterbags were collected and the leaves dried at room temperature (20°C) for 60 hr. The loss of weight after decomposition was 15% compared to senescent leaves.

A corresponding treatment was set up in which senescent leaves were repeatedly soaked in distilled water. It was assumed that soaking removes possible toxicity of the leaves but that the leaves have the same physical structure as unsoaked leaves. The soaked leaves were prepared in the following way. One hundred grams of dried bilberry senescent leaves were added to 1000 ml of distilled water and stirred for 8 hr. The extracts were decanted and another 1000 ml of distilled water was added, stirred at 8 hours, with the soaking process repeated 21 times in seven days. The leaves were then dried on plastic trays at 20°C.

Subsamples of 1.5 g dried leaf material and unlimed, unfertilized commercial peat, which served as control, were put in 50-mm Petri dishes, each filled with 10 ml distilled water. All Petri dishes were sealed with Parafilm and incubated in 20°C for 48 hr, so that all samples had the same moisture conditions at the start of the experiment. Pots (100 ml) were then half filled with sand (Silversand 90), and distilled water was added to saturation. The incubated leaves and peat were then placed on the sand surface and 10 pregerminated Scots pine and Norway spruce seeds, each with a radicle length of 1 mm, were placed on top of peat or leaves in each of the pots. All pots were covered with Parafilm and placed in a greenhouse at 20°C and with a day–night light ratio of 17:7. Pots were sprayed with distilled water daily. There were six replicates of each treatment for each species. The total numbers of rooted seedlings per treatment were counted daily for six days. A seedling was considered to be rooted when the radicle had penetrated the organic substrate and the cotyledons grew so that there was no contact with the organic substrate. The length (root plus hypocotyle) of each seedling was measured six days after the test had started.

RESULTS

Effects of Bilberry Aqueous Extract. Aspen seed germination was significantly reduced by all extracts compared to the distilled water control and the activated carbon treatment (Figure 1). The germination rates in the distilled

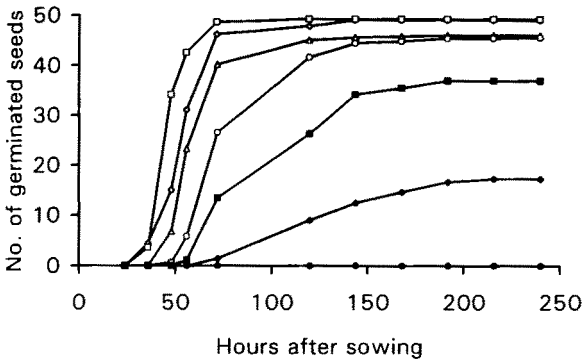

FIG. 1. Seed germination of aspen (*Populus tremula*) exposed to aqueous extract of bilberry (*Vaccinium myrtillus*) senescent leaves. Treatments: □, 10% extract treated with activated carbon; ◇, distilled water control; △, 2.5% extract, ▽, 5.0% extract; ■, 7.5% extract; ◆, 10.0% extract; and ●, 12.5% extract. See Table 1 for statistical analysis. $N = 5$.

water control and the activated carbon treatments were not significantly different (Table 1). Initiation of germination was delayed in all extract treatments when compared with germination in the distilled water control. In all extract concentrations, the root length of aspen was significantly reduced compared to the activated carbon treatment (Figure 2). Root length was reduced by 71% in the most diluted extract and by 97% in the 10% extract compared to the activated carbon treatment.

TABLE 1. ANALYSIS OF VARIANCE TABLE FOR EFFECTS OF BILBERRY (*Vaccinium myrtillus*) AQUEOUS EXTRACTS AND EXTRACTS TREATED WITH ACTIVATED CARBON ON SEED GERMINATION OF ASPEN (*Populus tremula*)[a]

Extract concentration	Hours										
	24	36	48	56	72	120	144	168	192	216	240
10% + act.carb.	a	a	a	a	a	a	a	a	a	a	a
Control	a	a	b	b	a	a	a	a	a	a	a
2.5%	a	b	c	c	b	b	b	b	b	b	b
5.0%	a	b	d	d	c	c	b	b	b	b	b
7.5%	a	b	d	e	d	d	c	c	c	c	c
10.0%	a	b	d	e	e	e	d	d	d	d	d
12.5%	a	b	d	e	f	f	e	e	e	e	e

[a]Control = distilled water. Treatments with the same letter within each column do not differ significantly in Tukey's multiple range test at $P \leq 0.05$. See Figure 1 for germination data.

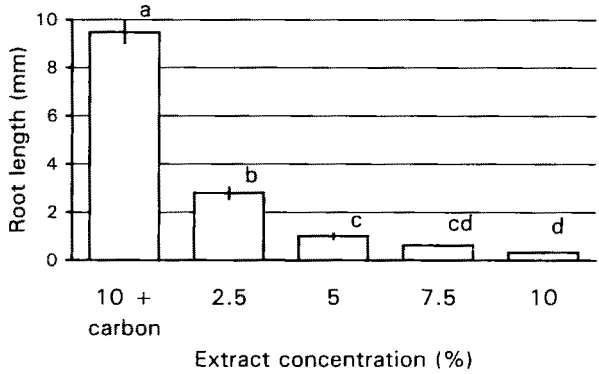

FIG. 2. Root length of aspen (*Populus tremula*) exposed to aqueous extracts of bilberry (*Vaccinium myrtillus*) senescent leaves and extract treated with activated carbon. Vertical line = standard error. Treatments with the same letter do not differ significantly in Tukey's multiple range test at $P \leq 0.05$.

Aspen roots in all extract treatments were necrotic, while those in the distilled water control and activated carbon treatments were fresh and normal in color (Table 2). All aspen seedlings in the 10% extract had abnormal prostrate growth forms (Table 2), while 49% of the seedlings had abnormal prostrate growth form in the most diluted solution. Seed germination of Scots pine and Norway spruce were also affected by the extracts, but less so than aspen. Scots pine germination was slightly (but not significantly) reduced in the 10% extract compared to the control (Table 3). Norway spruce germination was inhibited only by the 12.5% extract compared to the control (Table 3).

TABLE 2. PROPORTION OF ASPEN (*Populus tremula*) SEEDLINGS SHOWING ROOT NECROSIS AND PROSTRATE GROWTH IN AQUEOUS EXTRACTS OF SENESCENT BILBERRY (*Vaccinium myrtillus*) LEAVES[a]

Extract concentration	Root necrosis (%)	Prostrate grown (%)
10% + carbon	0	0.4
Control	0	3.7
2.5%	100	48.7
5.0%	100	77.5
7.5%	100	78.9
10.0%	100	100
12.5%	NS	NS

[a]Control = distilled water. NS = no seedlings available.

TABLE 3. SCOTS PINE (*Pinus sylvestris*) AND NORWAY SPRUCE (*Picea abies*) SEED GERMINATION IN AQUEOUS EXTRACT OF SENESCENT BILBERRY (*Vaccinium myrtillus*) LEAVES AFTER 14 DAYS[a]

Extract concentration	Germination (%)	
	Pine	Spruce
Control	100 a	100 a
2.5%	99.6 a	99.6 a
5.0%	91.5 a	98.8 ab
7.5%	92.4 a	100 a
10.0%	93.2 a	98.8 ab
12.5%	NS	83.0 b

[a]Numbers within columns followed by different letters are significantly different at $P \leq 0.05$ (Tukey's multiple range test). NS = no seedlings available. Control = distilled water.

Effects of Bilberry Senescent Leaves. Bilberry senescent leaves had significantly inhibitory effects on seed germination of Scots pine, Norway spruce, and birch when compared to the distilled water control (Table 4). Bilberry leaves delayed the start of germination by seven days for Scots pine and 10 days for Norway spruce and birch. After the seeds exposed to the leaves had been rinsed in water, germination increased from 24% to 93% for Scots pine and from 22% to 79% for Norway spruce (Table 4). Rinsing caused Scots pine germination to increase to the level of the control. Birch germination did not increase significantly after rinsing.

Seven days after sowing, the total germination of aspen was 80% in treatments with senescent bilberry leaves and 97% in the activated carbon treatment

TABLE 4. SCOTS PINE (*Pinus sylvestris*), NORWAY SPRUCE (*Picea abies*), AND BIRCH (*Betula pendula*) SEED GERMINATION IN SENESCENT BILBERRY (*Vaccinium myrtillus*) LEAVES AND DISTILLED WATER CONTROL AFTER 21 DAYS[a]

Treatment	Germination (%)		
	Scots pine	Norway spruce	Birch
Control	100 a	100 a	100 a
Senescent leaves	24.0 b	21.9 c	58.1 b
Rinsed seeds	92.6 a	79.1 b	66.6 b

[a]Rinsed seeds = germination 17 days after water cleaning treatment. Treatments with the same letter within each column do not differ significantly according to Tukey's multiple range test at $P \leq 0.05$.

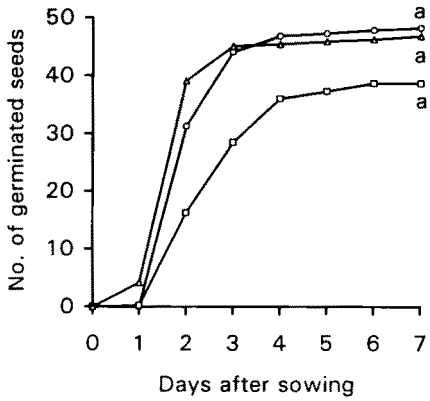

FIG. 3. Seed germination of aspen (*Populus tremula*) in senescent bilberry (*Vaccinium myrtillus*) leaves and leaves treated with activated carbon. ■, Senescent leaves; △, senescent leaves treated with activated carbon; and ○, distilled water control. Treatments with the same letter do not differ significantly in Tukey's multiple range test at $P \leq 0.05$, $N = 5$.

when compared to the distilled water control (Figure 3). None of the treatments differed significantly from the distilled water control.

Effects of Senescent and Decomposed Leaves on Root Development. The proportion of rooted Scots pine and Norway spruce seedlings was significantly reduced by both senescent and decomposed bilberry leaves compared to controls with peat and soaked leaves (Figure 4a and b). Senescent leaves were more inhibitory than decomposed leaves. Root growth was also significantly inhibited by senescent and decomposed bilberry leaves when compared to soaked bilberry leaves and peat (Figure 5a and b). The effects of the senescent leaves were the strongest, and these reduced root growth by 79% for pine and by 67% for spruce when compared to peat. Treatments with senescent bilberry leaves resulted in 17% mortality of Scots pine and 13% mortality of Norway spruce seedlings. No mortality occurred in treatments consisting of decomposed leaves, soaked leaves, or peat.

DISCUSSION

Bilberry litter has the potential to inhibit tree seedling establishment of the tested species, although these effects were weak for Norway spruce. Seed germination, growth, and root condition of aspen were negatively affected by even the most diluted extracts. Neither the pH nor the osmotic pressure of the bilberry

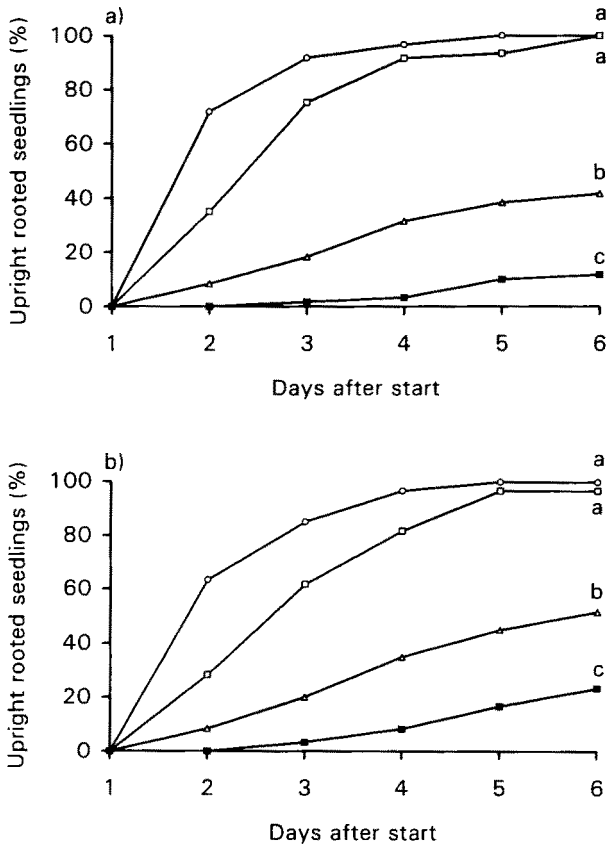

FIG. 4. Proportion of upright rooted (a) Scots pine (*Pinus sylvestris*) and (b) Norway spruce (*Picea abies*) seedlings in senescent and decomposed bilberry (*Vaccinium myrtillus*) leaves. Control = peat and soaked bilberry leaves. ■, senescent leaves; △, decomposed leaves; □, soaked leaves; ○, peat. Treatments with the same letter do not differ significantly in Tukey's multiple range test at $P \leq 0.05$.

water extract are likely to be responsible for the observed negative effects on seed germination we observed (Nilsson, 1992; Rikala and Jozetek, 1990).

The production and mode of action of phytotoxins present in senescent litter remains unknown. Our bioassays suggest that the inhibitory compounds are water soluble and released from senescent leaves during early decomposition. It is also possible that microorganisms could mediate transformation of compounds, causing inhibition both during the extraction process and later when the

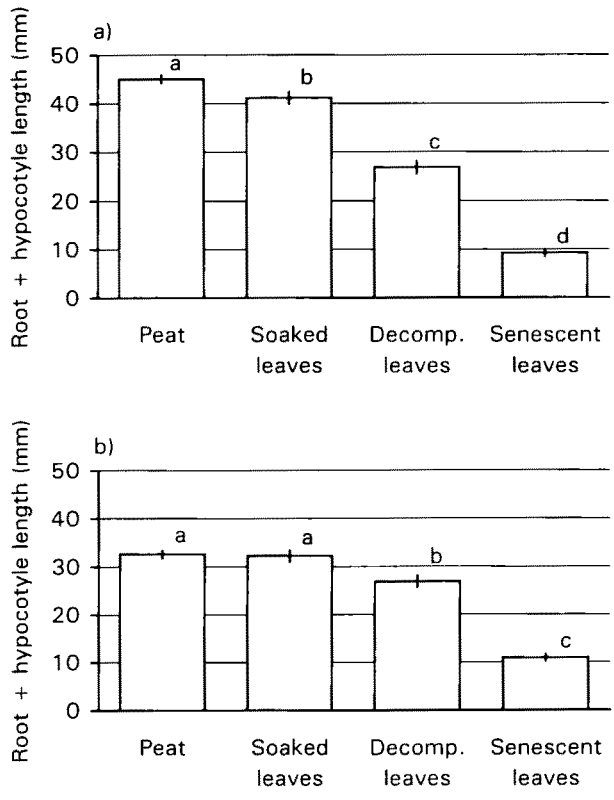

FIG. 5. Root and hypocotyle length of (a) Scots pine (*Pinus sylvestris*) and (b) Norway spruce (*Picea abies*) seedlings in senescent and decomposed bilberry (*Vaccinium myrtillus*) leaves. Control = peat and soaked bilberry leaves. Vertical line = standard error. Treatments with the same letter do not differ significantly in Tukey's multiple range test at $P \leq 0.05$.

water extracts were used in different bioassays. However the sterilization of extracts prepared from senescent leaves by 0.45-μm and 0.22-μm sterile filtration did not alter the degree of inhibition observed (data not shown).

Rinsing of exposed pine and spruce seeds removed the negative effects of water-soluble compounds extracted from bilberry litter. The results of the litterbag study with decomposing senescent leaves also indicates that when the litter produced in the autumn is allowed to decompose under subnival conditions during winter, between 34 and 74% of the inhibitory effect is lost. We interpret this to mean that natural rinsing of coniferous seeds and water-soluble compounds from bilberry litter by snowmelt and rain during normal field conditions

may reduce most of the inhibitory effects found in senescent litter. In experiments with *Empetrum hermaphroditum*, another dominant boreal species that forms clonal mats, Zackrisson and Nilsson (1992) found that the toxic compounds leached from leaf glands and absorbed by seeds could not be removed by washing exposed seeds. They also concluded that toxins were quickly absorbed by seeds and caused seed mortality before germination. The low seed mortality and radicle growth inhibition found in Scots pine and Norway spruce seedlings in our study indicates that the phytotoxic effects by bilberry litter are relatively limited, especially in comparison with results obtained in similar experiments with *Empetrum hermaphroditum* (Nilsson and Zackrisson, 1992).

The slower germination rate and radicle growth for seeds present in senescent leaves compared to that of the control treatments can be important for successful natural establishment of all species, but especially those with microbiotic seeds such as aspen. During the dry conditions prevailing in spring, the effect can be severe as radicle and whole seedlings are susceptible to drought and the litter may be more difficult to penetrate. Quick radicle penetration of litter and humus, which accelerates the opportunity of the seedlings with low nutrient resources to reach moist soil, can strongly reduce the susceptibility to water deficit under dry conditions (Grime and Curtis, 1976).

In all of our experiments, Norway spruce seeds and seedlings were less affected by bilberry litter and water extracts than were the other tree species. Norway spruce is a late successional climax species (Sirén, 1955; Havas and Kubin, 1983) and can be expected to be more tolerant of inhibitory effects from phytotoxic compounds than early successional species. However, it is also tempting to speculate that the lower level of inhibition found in spruce is an effect of coevolution between spruce and bilberry, since these two widespread boreal species may have coexisted in distinctive plant communities at least since the end of the Tertiary period (Walter and Breckle, 1989; Tallis, 1991). The deciduous broad leafed tree, *Populus tremula*, was more strongly inhibited by water extracts of senescent bilberry leaves than were the two coniferous species. *Betula pendula* seed germination in litter was relatively high, but rinsing had a much lesser revitalizing effect on these seeds than on the coniferous seeds following exposure to inhibitory compounds. Our interpretation is that the two boreal deciduous species we tested are more sensitive to litter of bilberry at the seedling stage than are coniferous species. Both aspen and birch are pioneer species that naturally colonize by seed in early successions after fire or other disturbances (Zackrisson, 1985; Grime et al., 1988). Fire has played an evolutionary important role in the maintenance of most boreal forest ecosystems dominated by bilberry (Sirén, 1955; Kuusela, 1990). Fire return intervals of less than 100 years have been determined for Norway spruce–bilberry forest types (Zackrisson, 1977). Nutritional changes (increased cation concentration and higher pH) caused by combustion of bilberry litter and organic soil following

fire (Viro, 1974; Pietikäinen and Fritze, 1995) can facilitate the establishment of pioneer deciduous trees such as aspen and birch. Species diversity also increases substantially in early postfire succession on previous bilberry sites (Schimmel, 1993). We postulate that this is not only a question of nutritional release, but also an effect of reduction of bilberry litter and humus that cause inhibitory effects on seedling establishment of early succesional species. In any ecosystem in which fire is a common element, one of its functions could be the destruction or deactivation of phenolics and other phytotoxic compounds found in litter and soil.

Addition of pure activated carbon in our bioassays resulted in significantly higher seed germination and seedling growth of aspen. These results support previous studies (e.g, Zackrisson and Nilsson, 1992; Mahall and Callaway, 1992; Yambe et al., 1992; Nilsson, 1994) and suggest that the activated carbon adsorbed inhibitory substances and deactivated them. The technique of using activated carbon to detoxify inhibitors therefore enables us to separate phytotoxic and nonphytotoxic effects. There is no reason to suppose that the carbon powder is itself a stimulatory factor in our experiments since distilled water amended with activated carbon did not cause stimulation of seed germination (Eliasson, 1959; Zackrisson and Nilsson, 1992).

It has previously been shown by Gallet and Lebreton (1995) that bilberry contains a range of phenolics including tannins, caffeic acid, *p*-coumaric acid, and flavonoids. Many of these also have been detected in high amounts in litter leachates from bilberry. These are all compounds with documented inhibitory effects on seed germination and seedling growth (Rice, 1984; Blum et al., 1984, 1985; Einhellig, 1987). Most of the phenolic compounds identified in bilberry have also been detected in soil (Blum and Rice, 1969; Whitehead et al., 1983; Kuiters and Danneman, 1987). Even if many of the phenolic compounds present are found only in low amounts in litter and soil, they can have an additive or synergistic effects on seed germination and growth (Rasmussen and Einhellig, 1977; Williams and Hoagland, 1982; Blum et al., 1985, 1989; Einhellig, 1987; Lyu et al., 1990; Lehman et al., 1994).

Our results provide evidence that phytotoxic effect of bilberry can be important in inhibiting seedling recruitment. However, these effects are not necessarily strong, and decomposing leaves do not appear to have a significant role in suppressing conifer tree seedling establishment. Our results also showed that the tree species we considered did not respond equally, and in particular spruce was less sensitive than the others.

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CHEMISTRY OF METAPLEURAL GLAND SECRETIONS OF THREE ATTINE ANTS, *Atta sexdens rubropilosa*, *Atta cephalotes*, AND *Acromyrmex octospinosus* (HYMENOPTERA: FORMICIDAE)

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Abstract—The chemical composition of the secretions of the metapleural glands of workers and soldiers of two *Atta* species, *Atta sexdens rubropilosa* and *A. cephalotes*, and workers of *Acromyrmex octospinosus*, has been studied. As indicated by infrared spectrometry and confirmed by the ninhydrin test, the secretions contain chiefly proteins. Of the volatile acidic portion, which is present as ionized salts, phenylacetic acid is the major component in workers and soldiers of *A. s. rubropilosa* and *A. cephalotes*. Both *Atta* species also contain 3-hydroxydecanoic acid and its homolog as minor components together with indoleacetic acid. While there are qualitative similarities in the acidic composition in the secretions of *A. s. rubropilosa* and *A. cephalotes*, they differ quantitatively. The secretion of *Acromyrmex octospinosus* contains 3-hydroxydecanoic and indoleacetic acids, but lacks phenylacetic acid. The bactericidal and fungicidal actions of the three major substances have been confirmed.

Key Words—*Atta sexdens rubropilosa*, *Atta cephalotes*, *Acromyrmex octospinosus*, metapleural glands, phenylacetic acid, Formicidae.

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INTRODUCTION

The metapleural glands of ants are located in the metathorax, where they open on either side through a permanently open duct adjacent to the hindleg coxae. The duct is internally connected with a large sclerotized bulla or reservoir, into which open the numerous secretory cells by means of their accompanying duct cells (Hölldobler and Engel-Siegel, 1984; Schoeters and Billen, 1993). Some functions, such as production of nestmate recognition substances, territorial-marking pheromones, protection against microorganisms (Maschwitz et al., 1970), and pollen germination inhibition (Sanderson and Wright, 1989) have been attributed to the secretion from these glands.

Work carried out some 20 years ago on the secretion from two myrmicine ants, using large amounts of insect material, identified phenylacetic acid (**1**) and indoleacetic acid (**3**) as components of the secretion of *Atta sexdens* and *A. s. rubropilosa* (Maschwitz et al., 1970; Schildknecht and Koob, 1970). Schildknecht (1976), using the metathoracic extracts of 40 ants, later identified three further components in the secretion of *A. s. rubropilosa*, namely 3-hydroxyhexanoic, 3-hydroxyoctanoic and 3-hydroxydecanoic acids. No further work was recorded on metapleural glands until recently, when three further reports appeared.

Attygalle et al. (1989) showed that the myrmicine species *Crematogaster deformis* has hypertrophied metapleural glands that contain 3-propyl and 3-pentylphenol, 5-propyl- and 5-pentylresorcinol and a related coumarin. Beattie et al. (1994) extended the claim for antibacterial action of the metapleural secretion to the Australian ant *Myrmecia gulosa*. Knapp et al. (1994) at the same conference, however, drew attention to the antifungal activity of the mandibular gland secretion of attine ants, while Jaffé et al. (1994) described the metapleural secretion of a large number of ant species as being composed largely of fatty acids. In the leaf-cutting species *Atta laevigata*, *Acromyrmex subterraneus*, and *A. landolti*, the major component was palmitic acid. They reviewed the claim for antimicrobial activity (Jaffé et al., 1994).

Independently, we have carried out studies to confirm the work of Schildknecht (1976), which up to this time had not been reexamined in the intervening two decades. Our objective was to work only with pure metapleural secretion and to avoid any contamination with other tissue or secretions. We have shown that the metapleural secretions of three attine species contain acidic compounds, as described by Schildknecht (1976), and the composition varies with species. The secretion also contains proteins. Microbiological tests confirm the activity of the secretion and synthetic substances against some bacteria and fungi.

METHODS AND MATERIALS

Colonies of *Atta sexdens rubropilosa* Forel, *A. cephalotes*, (L.) and *Acromyrmex octospinosus* (Reich.), from Vicosa and other locations in Brazil and Trinidad were maintained in laboratories at Leuven, Würzburg, or Exeter. Secretions from the metapleural glands of workers and soldiers of the two *Atta* species and workers of *A. octospinosus* were collected in fine glass capillaries (50–70 μm diameter) as described below. These capillaries were placed in large soft glass capillaries (1.8 $\pm$ 0.2 mm $\times$ 20 $\pm$ 5 mm), which had been first sealed at one end (Morgan, 1990). The larger capillaries were then completely sealed and transported to Keele for analysis.

To collect secretion, a system was devised that required a 10-μl syringe mounted in an expanded polystyrene block. The 50 to 70-μm capillary was drawn from a larger capillary in a flame, and the larger end was attached to the microliter syringe needle with a silicone tubing connection. The capillary was held steady in another small polystyrene block. The wall of the bulla of the metapleural gland was pierced with a fine insect-mounting pin, the ant was then held so the fine tip of the capillary are inserted into the hole in the bulla (Figure 1) and the secretion withdrawn into the capillary by gently raising the plunger of the syringe. With practice, this could be done routinely for a number of ants, collecting secretion from the paired metapleural glands in one capillary.

The infrared spectrum of the secretion of *A. sexdens rubropilosa* major workers was recorded with a FTIR Brucker microscope attached to a Brucker IFS 48 Fourier Transform infrared spectrometer. For the ninhydrin test, the section from one metapleural gland of each species was placed on filter paper and sprayed with 0.1% ninhydrin reagent (Merck) and heated to 110°C. Approximately equal quantities of hemolymph from each species were tested similarly.

The chemical analyses of these secretions were carried out by gas chromatography–mass spectrometry (GC-MS) with a solid-sampling technique (Morgan and Wadhams (1972). Preliminary analyses were made by directly crushing the sealed capillaries in the heated injection zone of the gas chromatograph and separating the components on a polar phase capillary column (polyethylene glycol, 12 m $\times$ 0.20 mm ID $\times$ 0.25-μm film thickness). This method did not give very satisfactory results for the very small quantities of acidic secretion. We had to modify the technique by opening each capillary at the tip, converting the acids to their methyl esters by adding a solution of diazomethane in ether (50 μl), and blowing off the ether with a stream of nitrogen, then resealing the capillary and using the solid-sampling method. These methyl esters were then analyzed on a column with a nonpolar phase (dimethylsiloxane, 12 m $\times$ 0.22 mm ID $\times$ 0.25-μm film thickness). The oven was programmed from 30°C at

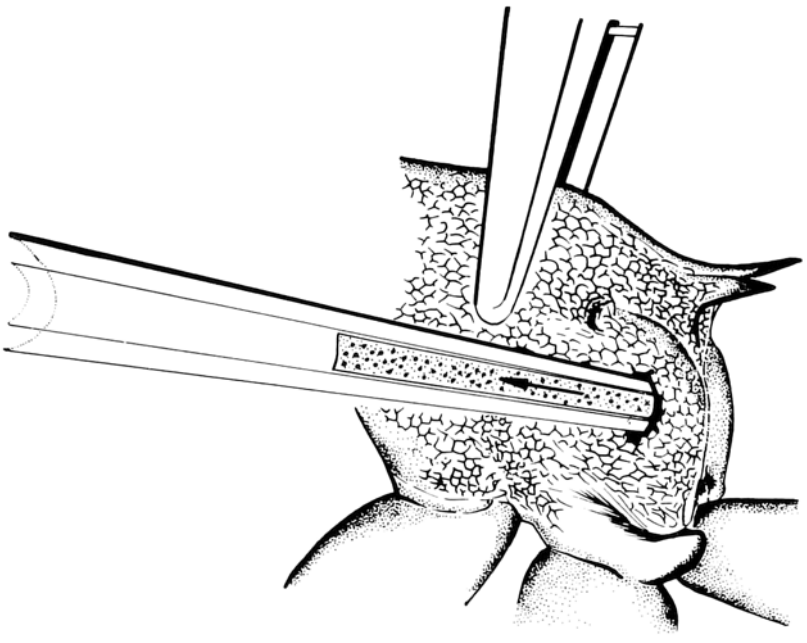

FIG. 1. Method for removing metapleural gland secretion from an ant. After perforating the reservoir cuticle with an insect pin, the capillary was inserted into the opening. The ant was held with fine forceps. Arrow indicates secretion transport inside the capillary.

8°C/min to 250°C and helium was used as the carrier gas at 1 ml/min. The split vent was closed before crushing the sample and reopened 30 sec later. The chemical substances separated were identified by interpretation of the mass spectra of their methyl esters and confirmed by comparison of retention time and mass spectrum with synthetic standards. The analyses were carried on at least two colonies of each species, using at least 10 individual specimens for each colony caste. The methyl esters of phenylacetic and indoleacetic acids were prepared by esterification of their corresponding acids with diazomethane, and the methyl esters of the 3-hydroxy acids were obtained via a Reformatsky reaction. These compounds were used as external standards for quantification of the components, and for microbial assays.

Preparation of Methyl 3-Hydroxy Esters. Methyl 3-hydroxy esters were prepared from a mixture of methyl bromoacetate (2 ml, 20 mmol, Fluka) and either hexanal, octanal, or decanal (20 mmol, Aldrich) dissolved in a mixture of dry toluene and ether (4 : 1). The solution was added dropwise to zinc powder

(2 g, 20 mmol) and the mixture was refluxed for 1.5 hr, giving the zinc alkoxide of the hydroxy-ester. This was treated with a solution of sulfuric acid (10%), followed by aqueous sodium carbonate (10%). The product was extracted with diethyl ether (50 ml) and the solvent layer was dried ($CaSO_4$), filtered, and the solvent removed by rotatory evaporation to yield the methyl esters of 3-hydroxyhexanoic, -octanoic, -decanoic and -dodecanoic acids, respectively. The yield in each reaction was approximately 60%.

Microbiological Tests. To test for the inhibition of spore germination by metapleural gland secretion of *A. s. rubropilosa* the following fungi were used: *Botrytis cinerea*, *Pyricularia oryzae*, *Trichoderma viride*, and *Venturia inaequalis*. The spores were stored in a refrigerator and subjected to heat-shock immediately before use to induce germination. Spore suspension (10 μl) was transferred into sterile potato dextrose bouillon (2.4%) with glucose (1%) (10 ml, diluted 1 : 2) containing tetracycline (final concentration 0.05%). An aliquot of this suspension (80 μl) was placed in a microliter plate either with (experimental) or without (control) metapleural gland secretion (1–2 μl) and maintained at 25°C for 33 hr. The samples were observed by light scattering in a spectrophotometer at 595 nm.

For antibacterial tests, the following species were used: *Bacillus cereus* (6923, numbers in the Difco catalogue), *Bacillus sphaericus* (7134), *Pseudomonas fluorescens* (1794), and *Pseudomonas putida* (2257). The bacterial were grown on Muelder-Hinton agar (Difco). To inoculated Petri dishes were added either metapleural gland secretion (1 μl) or an aqueous suspension of the synthetic acids in distilled water (1 : 1) (1 μl).

RESULTS

The milky white secretion from the metapleural glands can be collected in a fine glass capillary as reported by Schildknecht (1976), but can be collected more easily by first piercing the bulla (Figure 1) and then inserting the capillary into the orifice. We first examined a droplet of the secretion of *A. s. rubropilosa* with the aid of a microscope and a Fourier transform infrared spectrometer. The spectrum showed very broad OH and possibly NH absorption with a sharp maximum at 3287 cm^{-1} and a secondary maximum at 3086 cm^{-1}. There was no carboxylic acid C=O absorption near 1700 cm^{-1}. There were two strong bands at 1655 cm^{-1} and 1626 cm^{-1}, which we attributed to amide I and II, and a broad absorption at 1549 cm^{-1}, which can be attributed to ionized carboxylate (COO^-) (Figure 2). We concluded that the spectrum showed chiefly the characteristics of a concentrated peptide or protein solution. This was confirmed for each species using the ninhydrin test for protein, which gave clear positive tests.

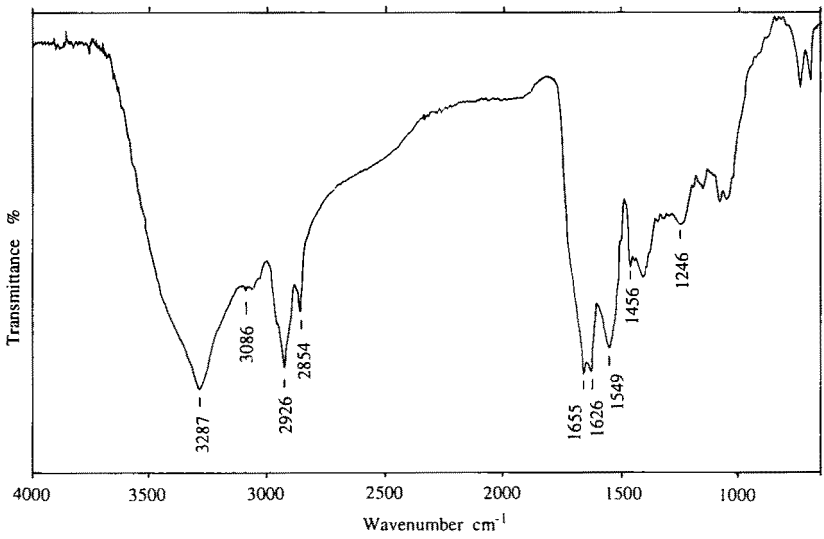

FIG. 2. Infrared spectrum of the total metapleural gland secretion obtained from a major worker of *Atta sexdens rubropilosa*.

For comparison, the same quantity of hemolymph from an ant gave a weaker reaction.

Phenylacetic acid (Figure 3, **1**) was identified in the secretion of *A. s. rubropilosa* on a polar phase capillary gas chromatography column. However, converting the free acids into their corresponding methyl esters with diazomethane showed the presence of other acids in the secretions (Figure 4). The second most important constituent was 3-hydroxydecanoic acid. The exact composition varied with the individual, as indicated by the large standard deviations in Table 1. The soldiers showed a greater proportion of phenylacetic acid than the workers, but no more secretion. There were no differences found between colonies. Schildknecht and Koob (1970) found on average 1.4 μg of phenylacetic acid per insect. We found a mean of 0.54 μg per major worker, and 0.82 μg per soldier. The difference may suggest incomplete removal of secretion by our method.

In *A. cephalotes*, phenylacetic acid and indole acetic acid were the major substances (Figure 5). Again there were differences in composition between soldiers and workers, the soldiers having more secretion than we found in the workers. The secretion of *A. cephalotes* contained traces of the odorous compound skatole (**4**), which was not detected in *A. s. rubropilosa*, while the 3-hydroxy acids were undetected in workers.

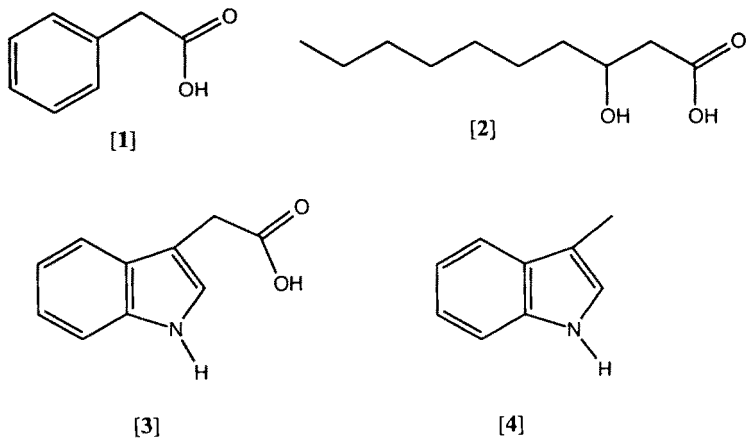

FIG. 3. Structures of the compounds found in the metapleural glands: **1:** phenylacetic acid; **2:** 3-hydroxydecanoic acid; **3:** indoleacetic acid; **4:** skatole.

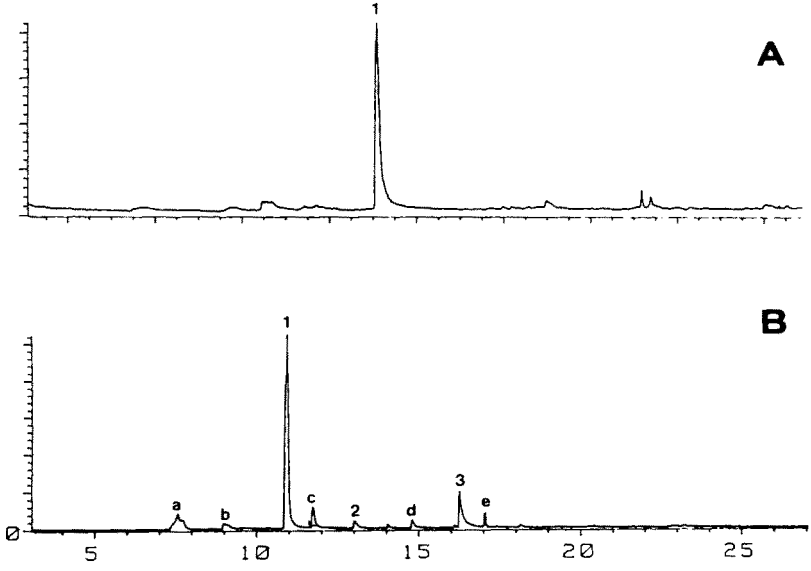

FIG. 4. Total ion chromatogram of the volatile acids (as their methyl esters) from the metapleural glands of *Atta sexdens rubropilosa*. (A) From a major worker: (B) from a soldier caste. Peaks a–e are impurities introduced with the diazomethane solution.

TABLE 1. CHEMICAL COMPOSITION OF VOLATILES IN METAPLEURAL GLAND SECRETION OF *Atta sexdens rubropilosa* WORKERS AND SOLDIERS (N = 12)

Compound number in Figure 4	Compound[a]	Mean proportion (% ± SD)	
		Workers	Soldiers
1	Phenylacetic acid	57.3 ± 24.5	86.5 ± 13.4
2	3-Hydroxyoctanoic acid	t	
3	3-Hydroxydecanoic acid	35.9 ± 24.9	12.0 ± 12.6
4	3-Hydroxydodecanoic acid	6.85 ± 5.6	1.5 ± 3.2
	Indoleacetic acid	t	t
Mean total amount (μg)		0.95 ± 1.2	0.95 ± 1.4

[a]The compounds were identified as their methyl esters after derivatization with diazomethane.

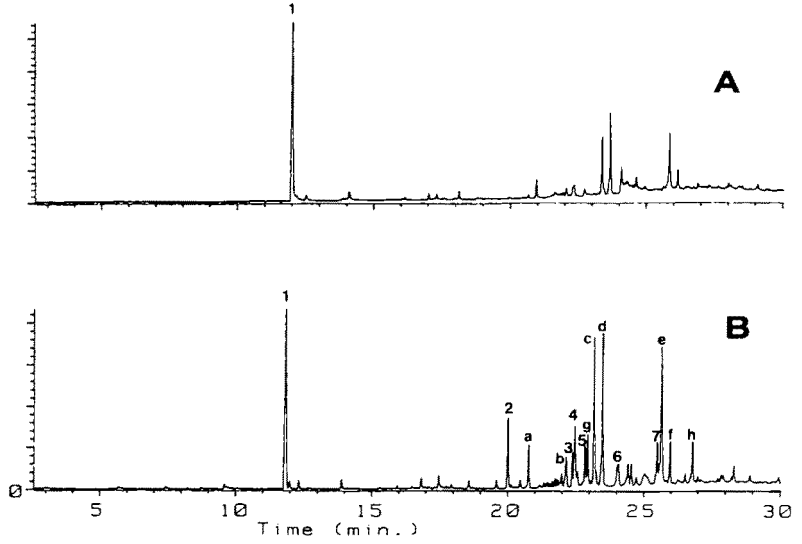

FIG. 5. Total ion chromatogram obtained after treatment with diazomethane of the volatile part of the metapleural gland secretion of *Atta cephalotes*. (A) Major workers, (B) soldier. For numbered peaks, see Table 2. Peaks a–f are fatty acid esters ranging from C_{12} to C_{18} that were found to be contaminants in the diazomethane solution and peaks g and h are hexadecane and octadecane, respectively.

The metapleural gland secretions of *A. octospinosus* were characterized by the presence of 3-hydroxydecanoic acid (**2**) and indoleacetic acid (**3**) and by the absence of phenylacetic acid (Figure 6). Again no colony differences were found.

In tests on the phytopathogens, *Botrytis cinerea* showed germination and hyphal growth in both the experimental and control conditions. *Pyricularia oryzae* showed abundant mycelial growth in both control and experimental conditions, while *Trichoderma viride* showed strong mycelial growth in the control, but no germination in the experimental conditions. *Venturia inaequalis* showed only slight reduction of spore germination in the presence of the metapleural gland secretion.

For antibacterial tests, the secretion of minor, medium, and major workers of *A. s. rubropilosa* was placed on an agar plate together with phenylacetic acid, 3-hydroxydecanoic acid, and indoleacetic acid. The metapleural gland secretion showed strong growth inhibition against *Bacillus sphaericus* and *Pseudomonas putida* and a weaker effect against *B. cereus* and *P. fluorescens* (Figure 7). It showed little effect against *E. coli* and *S. aureus*. Phenylacetic acid in the pure state was inhibitory towards all six species, and 3-hydroxydecanoic acid was inhibitory towards all but *S. aureus*. Indoleacetic acid, which tended to diffuse into the agar and gave larger, more diffuse spots, was strongly inhibitory

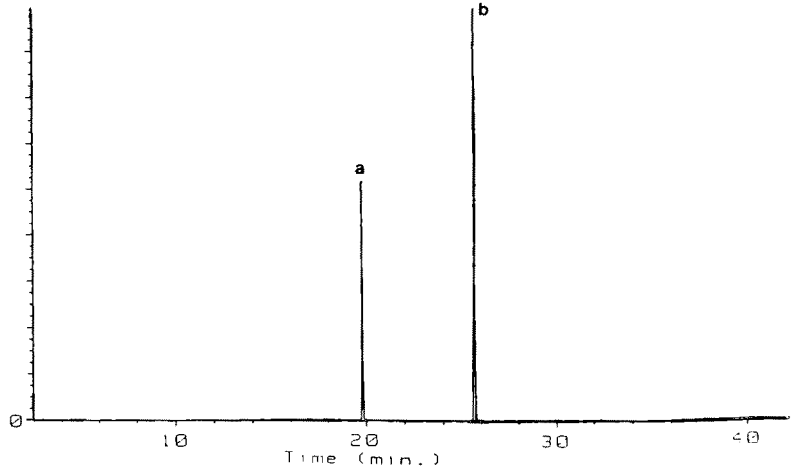

FIG. 6. Ion chromatogram of *m/z* 103 for the metapleural gland secretion of an *Acromyrmex octospinosus* worker, after treatment with diazomethane. Peak a, 3-hydroxydecanoic acid; b, indoleacetic acid (as their methyl esters). The amount of secretion was very small and the peaks did not show up well in a total ion chromatogram.

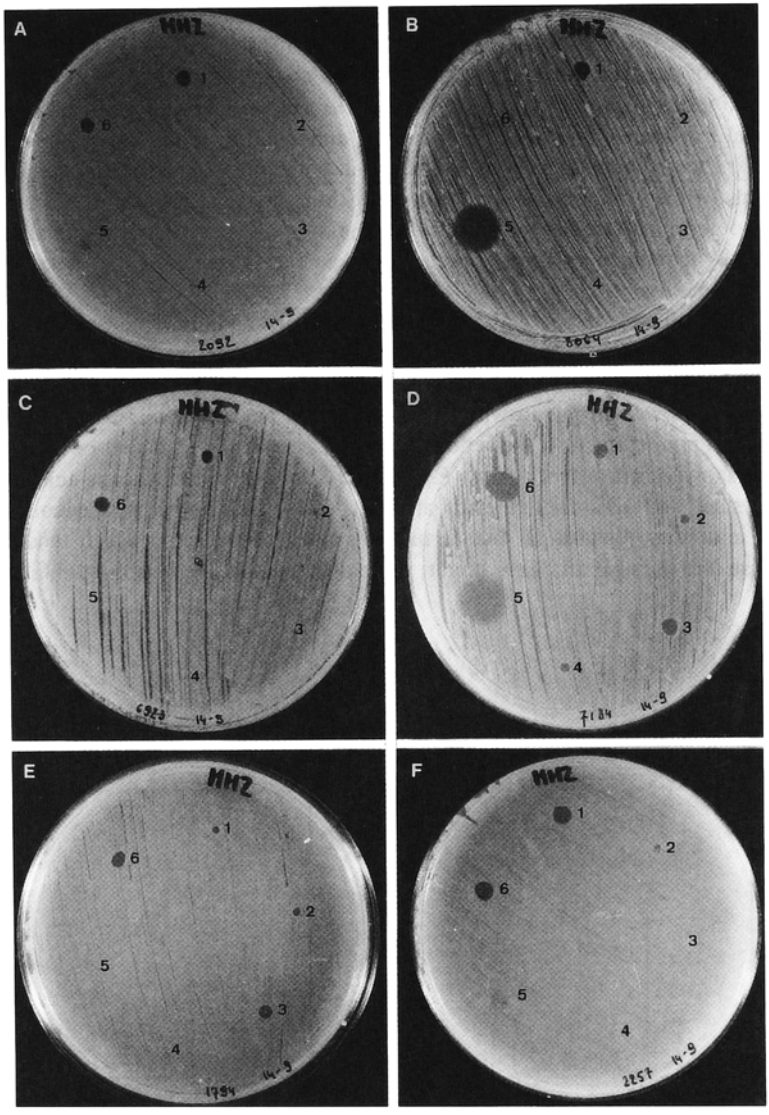

FIG. 7. Results of inhibition of bacterial growth. An emulsion of phenylacetic acid and distilled water, 1 : 1 (1); 1 μl metapleural gland secretion of *A. s. rubropilosa* major (2); medium (3) and minor (4) workers were placed on the agar plates (MHZ) together with indoleacetic acid and water 1 : 1 (5); and 3-hydroxydecanoic acid and water 1 : 1 (6). (A) *Escherichia coli*; (B) *Staphylococcus aureus*, (C) *Bacillus cereus*; (D) *Bacillus sphaericus*, (E) *Psuedomonas fluorescens*, (F) *Pseudomonas putida*.

toward *S. aureus* and *B. sphaericus*, weakly inhibitory towards *P. putida* and *E. coli*, and showed no effect against *B. cereus* and *P. fluorescens*.

DISCUSSION

The metapleural gland secretion of the three species of attine ant examined here is a milky white substance, and is thus an emulsion, not a solution. The secretion is readily seen through the thin cuticle of minor and medium workers of *A. s. rubropilosa*, so that individuals with full reservoirs can be identified, but it is not visible through the more opaque cuticle of major workers and soldiers.

The infrared spectrum of the secretion of *A. s. rubropilosa* was not at all like that of a mixture of carboxylic acids. There was no evidence of carboxylic acid C=O absorption of phenylacetic acid (at 1697 cm^{-1}) or of the 3-hydroxydecanoic acid (at 1730 cm^{-1}). It showed strong OH absorption, typical of associated hydroxy groups, which could be a concentrated aqueous solution. It showed a sharp maximum at 3287 cm^{-1}, typical of secondary amides, and two very strong bands at 1655 cm^{-1} and 1626 cm^{-1}, which could be attributed to amide I and II bands of peptides or proteins. The broad band at 1549 cm^{-1} can be correlated with ionized carboxyl. We concluded that the secretion was an aqueous emulsion of protein and the ionized form of the carboxylic acids. The secretion gave a strong, positive ninhydrin test for protein, stronger than that of hemolymph. Schoeters and Billen (1993) have already described the glandular endoplasmic reticulum in the secretory cells of the metapleural gland, which suggests considerable protein-synthesizing ability. The remainder of the study concentrated on the volatile part of the secretion.

Carboxylic acids are not ideal compounds for gas chromatography because they are very polar and small quantities can be lost through adsorption on surfaces in the instrument. Initially, we showed that phenylacetic acid was present in the secretion of *A. s. rubropilosa*. It gave a clear peak when the whole secretion was chromatographed on a polar column, but no other substances were visible. Thereafter, we converted all carboxylic acids to their less polar methyl esters by treating the secretion on a microscale with diazomethane solution. Minor peaks were then visible, which from their mass spectra and coincidence of retention times were identified as the compounds listed in Tables 1 and 2. While Schildknecht and Koob (1970), Maschwitz et al. (1970), and Schildknecht (1976) apparently were unaware of the presence of protein in the secretion, we confirmed the report of Schildknecht (1976) that the major volatile and acidic substances in *A. s. rubropilosa* were phenylacetic acid followed by 3-hydroxydecanoic acid, which we synthesized for comparison. However, where Schildknecht found the C_6, C_8, and C_{10} β-hydroxyacids, we found the C_8, C_{10}, and

TABLE 2. CHEMICAL COMPOSITION OF VOLATILES IN METAPLEURAL GLAND SECRETION OF *Atta cephalotes* WORKERS AND SOLDIERS (N = 15)

Compound numbers in Figure 5	Compound[a]	Mean proportion (% ± SD)	
		Workers	Soldiers
1	Phenylacetic acid	99.3 ± 1.7	63.2 ± 26.1
	Skatole	0.1 ± 0.4	0.9 ± 1.6
2	3-Hydroxydecanoic acid		
3	4-Hydroxyphenylacetic acid	t	
4	3-Hydroxydodecanoic acid		6.1 ± 7.1
	Unknown 3-hydroxy acid I		2.7 ± 4.4
5	3-Hydroxytetradecanoic acid		4.1 ± 4.7
6	3-Hydroxyhexadecanoic acid		3.8 ± 7.1
	Unknown 3-hydroxyacid II		0.1 ± 0.3
	Indoleacetic acid	0.6 ± 1.7	18.4 ± 9.9
Mean total amount (μg)		0.01 ± 0.03	0.2 ± 0.2

[a]The compounds were identified as their methyl esters after derivatization with diazomethane.

C_{12} β-hydroxy acids. The mean composition of the large workers' and soldiers' secretions were different, but because of the wide individual variations, it is not clear if this difference is important.

Microbiological tests showed that the antibacterial action of the secretion can be ascribed to the volatile acid portion. However, as pointed out by Hölldobler and Engel-Siegel (1984), the presence of an antiseptic effect in the secretion does not necessarily mean that the primary function of the gland is antisepsis. The work of Van den Broeck (1993) and Knapp (1994) showed that there are powerful antiseptic effects in the mandibular glands of attine ants.

According to Diehl-Fleig and Valim-Labres (1993), isolation of *Aspergillus* spp., *Mucor* spp., and *Penicillium* spp. from *Atta sexdens piriventris* and *Acromyrmex heyeri* workers shows that mandibular and metapleural gland secretions are not fungicidal to at least some species. These results are also supported by the fact that one can find fungus ants with *Beauveria bassiana* (entomopathogen) (Diehl-Fleig et al., 1992) and *Metarhizium anisopliae*. Thus, it seems that the ants cannot avoid the occurrence of alien fungi.

In preliminary tests, Van den Broeck (1993) found that the Atta garden fungus (*Attamyces* sp.) did not inhibit germination of *Trichoderma hamatum* spores. *T. hamatum* and *T. viride* are mycotoxic fungi. We, therefore, concluded there was no natural protection against pathogenic fungi in the fungus garden, and included *T. viride* in our tests. Strong hyphal growth of *T. viride* was

observed in the control, but no growth at all occurred in the experiment. This indicated that the metapleural substances could provide protection for *Attamyces* from pathogens. Powell and Stradling (1986) have already shown that the metapleural gland secretion had only a minor influence on the growth of *Attamyces*.

The metapleural gland secretion of *A. cephalotes* workers contains phenylacetic acid, and the secretions from the soldiers contain four β-hydroxy acids (C_{10}, C_{12}, C_{14}, and C_{16}). In addition, the soldiers contained two unidentified compounds, which after conversion to methylesters, had mass spectra that showed a base peak of m/z 103 and fragmentation patterns similar to unsaturated hydrocarbons. This is characteristic of methyl β-hydroxyesters. We therefore concluded that these are from unsaturated β-hydroxy acids. Skatole, not detected in *A. s. rubropilosa*, was also present in *A. cephalotes* and is probably responsible for the distinct odor of the secretion.

The secretion of *Acromyrmex octospinosus* workers was more difficult to collect and different from that of the two *Atta* species. By searching the mass spectra, 3-hydroxydecanoic acid and indoleacetic acid were identified and confirmed by selective ion monitoring. *A. octospinosus* secretion did not contain phenylacetic acid. It is noteworthy that Schildknecht and Koob (1970) also recorded that *Acromyrmex subterraneus* lacked phenylacetic acid. Indoleacetic acid (a heteroauxin) is well known as a plant growth hormone.

It is clear that these leaf-cutting ants possess a metapleural gland secretion that contains, in part, volatile acids that have antibacterial action. They could protect the ant fungus garden from other fungi, which, like *Trichoderma viride*, produce mycotoxins. The earlier work of Maschwitz et al. (1970) and Schildknecht (1976) are confirmed to that extent. However, the gas chromatography, powerful as it is, is not the solution to all analytical problems, and it cannot detect peptides and proteins. To obtain a solution in an organic solvent and to analyze the volatile components of that solution may be to overlook other substances. Unless the secretion is homogeneous and the components identified can account for the full mass of the secretion, such results must be treated with caution. The metapleural gland secretions of these ants require further investigation before we know their full composition and function.

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STABLE FREE RADICAL FROM PLANT LITTER DECOMPOSING IN WATER

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Abstract—The presence of a stable radical species in both fresh straw and that which had been submerged in aerated water for up to six months has been demonstrated using electron paramagnetic (spin) resonance (EPR or ESR) spectroscopy. A radical signal was associated also with material shown to contain straw lignin markers, which was leached from the rotting straw into surrounding water. Fresh straw treated with strong alkali to remove phenolics did not show a radical signal. The possible effect of a dissolved stable free radical is discussed in relation to the antagonistic effect of rotting straw on algal and cyanobacterial growth in water to which straw has been added as a nuisance algal control agent.

Key Words—Electron paramagnetic resonance, electron spin resonance, stable free radical semiquinone radical, aquatic decomposition, soluble lignin, algal control.

INTRODUCTION

A free radical is simply any species that contains one or more unpaired electrons. The resulting paramagnetism of radicals can be detected using electron paramagnetic resonance (EPR) spectroscopy, providing that the radical persists for long enough to permit analysis. While highly reactive free radicals currently are receiving unprecedented attention (Halliwell et al., 1995), possibly because they

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are implicated in the aging process and a range of human diseases, few studies have been directed towards the effect of radicals that are long-lived.

Remarkably stable organic free radicals have been detected in biological polymeric material including lignin, tannin, and melanin (Tolin and Steelink, 1966; Senesi et al., 1987). A stable free radical is present in senescent plant material and its occurrence is possibly ubiquitous in plants (Hendry et al., 1994). Atherton et al. (1993) postulated that the radical is derived from a quinone, although its provenance is unclear. It is likely that radicals in biopolymers are protected by entrapment within the macromolecule (Steelink, 1966) and, as biopolymers are generally recalcitrant in the natural environment, little consideration has been given to the possible reactivity of such radical sites.

Lignin, of course, is recognized as one of the most recalcitrant fractions of plants. However, it has been reported previously that portions of the lignin macromolecule are solubilized following microbial degradation of lignocellulosic substrates under laboratory conditions (Nordkvist et al., 1989; Suzuki et al., 1990) including barley straw (Zimmerman and Broda, 1989; Kivaisi et al., 1990). Dissolved macromolecular material was recovered from aqueous solution either by acid (Crawford et al., 1983) or solvent (Suzuki et al., 1989) precipitation; the soluble lignin material was shown to be of medium molecular weight (Crawford et al., 1983). We have confirmed, using pyrolysis gas chromatography–mass spectrometry (GC-MS), the presence of lignin markers in macromolecular material recovered by ethanol precipitation from solution in the water containing barley straw rotting under natural conditions (Pillinger, 1993; Pillinger and Gilmour, unpublished data).

Our aims here are twofold: (1) to confirm that barley straw, and in particular the lignin fraction, shows an EPR spectrum characteristic of an organic free radical, and (2) to determine whether the free radical is transferred to the aqueous environment in association with macromolecular lignin solubilized during microbial degradation.

METHODS AND MATERIALS

Barley, *Hordeum vulgare*, straw was obtained from a local farm and stored in a dry barn. About 2 g dry wt straw to each liter of aged tap water was subjected to decomposition under water either in a tank of well-aerated water as described previously (Pillinger et al., 1994) or in a shallow experimental pond (ca. 1000-liter capacity). In the latter case, straw (ca. 60 g dry wt) was contained in mesh bags suspended just below the surface to maximize natural aeration. The straw appeared to retain structural integrity for at least six months, although it often became discolored due mainly to fungal infection. Rotted straw for the studies reported here was harvested from either the tank or pond. Unrot-

ted straw was subjected to alkali treatment by immersion in 1 M NaOH overnight followed by thorough rinsing. Details of the various straw samples and treatments are summarized in Table 1.

A precipitate, which included polyphenolic material (soluble lignin), was prepared from either the culture medium or from pond water in which straw had soaked. The solution was passed through a glass fiber filter (1.2-μm pore size) and typically a 50-ml aliquot was added slowly to a fivefold excess of 99% ethanol (Suzuki et al., 1989). The precipitate was recovered by centrifugation and dried under nitrogen.

A preliminary and rapid method to screen for the presence of radical sites is afforded by X-band EPR spectroscopy. A single broad signal is indicative of a radical site, but in order to resolve the signal further, Q-band EPR spectroscopy, which provides higher resolution of radical signals due to higher spectrometer frequency, is required. X-band EPR spectra were recorded on a Varian E109 spectrometer operating with a klystron tuned to ~9 GHz. In X-band, a modulation amplitude of 0.2 mT, significantly less than the observed radical line width, was used. The Q-band EPR spectra were recorded on a Varian E112 spectrometer at ~35 GHz using a modulation amplitude of 0.5 mT, again not greater than the line width of the EPR signals. All EPR spectra were obtained at room temperature.

Internodal sections only of straw were used for EPR spectroscopy. Fresh straw and alkali-treated samples were analyzed without additional treatment. A

TABLE 1. DESCRIPTION OF SAMPLES

Sample	Preparation of sample prior to EPR spectroscopy
1. Unrotted straw	Barn-stored straw removed to laboratory and stored at room temperature
2. Rotted straw (tank)	Straw soaked in laboratory tank for six months, sample removed, soaked in algal culture medium for two weeks at room temperature
3. Rotted straw (tank)	As for sample 2, but straw air dried for further seven days at room temperature
4. Rotted straw (pond)	Straw soaked in pond for two months, straw sample removed and soaked in same pond water for two weeks at room temperature
5. Alkali-treated straw	Unrotted straw (sample 1) immersed in 1 M NaOH overnight
6. Soluble lignin from aqueous solution containing sample of 2.	Precipitate prepared from the algal culture medium in which sample 2 was soaked
7. Soluble lignin from aqueous solution containing sample of 4.	Precipitate prepared from pond water in which sample 4 was soaked

sample of rotted straw (250 mg) was soaked further in 250 ml algal culture medium at pH 8 (historically used in experiments designed to test the algal toxicity of rotted straw) (Pillinger et al., 1994) or pond water (250 ml) for two weeks prior to analysis. The sole purpose of the additional period of laboratory incubation of naturally decomposed straw from the pond was to concentrate the straw leachate in the aqueous phase for subsequent preparation of sufficient soluble lignin fraction for analysis; tank-rotted straw was treated similarly for comparison. Initially any wet straw samples were blotted dry; such drying proved adequate for X-band spectroscopy, although subsequent measurements (and Q-band spectroscopy) were carried out on samples that had been air dried in the laboratory. Straw, approximately 6 mg/cm vertical depth of cavity, was inserted into a 3-mm quartz EPR tube; no attempt was made to relate the strength of radical signal with the amount of sample scanned. During each batch of analyses, a spectrum of the standard, diphenylpicrylhydrazine (DPPH), was recorded. The values of g, which characterize the EPR signals, are calculated relative to the known g value for DPPH. An uncertainty in the estimation of the g value arises due to the signal-to-noise ratio and lack of symmetry of the signal and due to overlapping signals particularly in the Q-band spectra. In order to take account of such uncertainity, g values are quoted as falling within a specified range.

RESULTS

Under the operating conditions of field sweep and signal-to-noise ratio, the g value for the X-band signals are quoted to within ± 0.0005 (Table 2). Similar values for the Q-band spectra are quoted in Table 3. Repeated scanning (typically three times) of each X-band spectrum prior to the recording showed that the reproducibility of any one spectrum was within the range that the spectroscopic analysis imposes on the g values. Likewise, replicate subsamples ($N = 3$) of pond-rotted straw analyzed sequentially and samples of tank-rotted straw ($N = 3$) analyzed at different times during a three-month period had g values in the stated range. The data quoted below, therefore, represent the values of g obtained on individual representative samples. The significant features of the data are, first, the presence or absence of a signal and, second, those parameters that indicate the presence of an organic free radical (a g value close to 2 and the shape and line width of the signal).

Qualitative X-band electron paramagnetic resonance spectroscopy indicated that a radical was present in each of the samples studied, with the exception of the straw that had been delignified by alkali treatment (Table 2). The radical was similar in the samples analyzed both in terms of the g value and line shape and width (ca. 0.5 ± 0.05 mT) and was close to the free-spin value ($g \sim 2$)

TABLE 2. DESCRIPTION OF X-BAND EPR SIGNAL RECORDED FOR VARIOUS STRAW SAMPLES

Sample[a]	g[b]
1. Unrotted straw	2.0048
2. Rotted straw (tank)	2.0037
3. Repeat of 2 after further drying	2.0046
4. Rotted straw (pond)	2.0041
5. Alkali-treated unrotted straw	na[c]
6. Soluble lignin recovered from aqueous solution containing sample of 2	2.0041
7. Soluble lignin recovered from aqueous solution containing sample of 4	2.0047

[a] See Table 1 for details.
[b] g values calculated relative to the g value for diphenylpicrylhydrazine (DPPH).
[c] Signal absent.

TABLE 3. Q-BAND EPR SPECTROSCOPY OF SELECTED SAMPLES

Sample[a]	Radical signal[b]	g[c]	Approx. relative strength[d]
1. Unrotted straw	a	2.0046	+ + + + +
	b		
	c	2.0019	+
3. Rotted straw (tank)	a	2.0049	+ + + + +
	b	2.0033	+ +
	c	2.0022	+
4. Rotted straw (pond)	a	2.0053	+ + +
	b	2.0034	+ + + + +
	c	2.0025	+
7. Soluble lignin	a	2.0042	+ + + + +
	b		
	c	2.0019	+

[a] See Table 1.
[b] See Figure 2.
[c] The g values for signals a and c were estimated from the peak turnover (maximum) and for signal b from the inflection point. Uncertainties in g values are about ± 0.0005 for peaks a and c and ± 0.0002 for peak b.
[d] Relative strength within a spectrum; quantitative comparison between samples was not made.

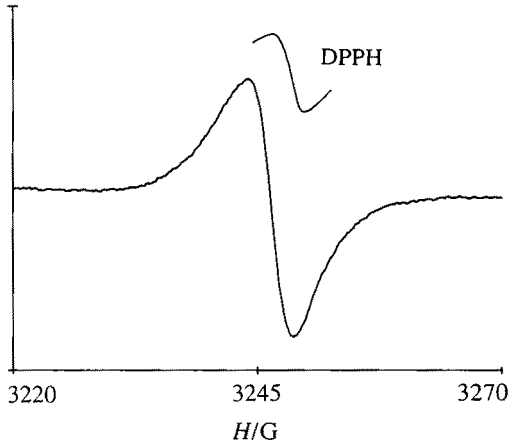

FIG. 1. X-band EPR spectrum of pond-rotted straw. The X-band EPR spectrum (9.1 GHz) shown was recorded on an air-dried sample of pond-rotted straw (sample 4, Tables 1 and 2). DPPH standard included in the sample cavity, shown as insert spectra overlap.

expected for an organic free radical. The X-band EPR spectrum of pond-rotted straw is shown in Figure 1.

A further examination of some of the samples by Q-band EPR spectroscopy established that power saturation occurred in the range of 10–20 dB, confirming the radical nature of the signal. The Q-band spectra were more informative than the X-band ones and revealed that, although the material was broadly similar in all cases, different radical signals with differing intensities were present (Table 3). Thus, fresh straw had two major signals, one with a g value of 2.0046 (Table 3, signal a), which was some five times greater in intensity than the other (c) with a g value of 2.0019. The radical signals a and c were present in both of the rotted straw samples, but in addition, a further signal b with an intermediate g value of 2.0033 was present. In the spectrum of the pond-rotted straw, this signal was the most intense of the three signals. The Q-band EPR spectra of unrotted and pond-rotted straw are shown in Figure 2. The spectrum of the latter sample clearly shows the presence of an additional signal (b) of intermediate g value. The soluble lignin preparation showed a spectrum that was essentially the same as that of the fresh straw, both in terms of g values and relative intensity of the radical signals a and c. No signal corresponding to b was observed in the soluble lignin preparation. We conclude that signal b derives from a different radical site than signals a and c. Signals a and c, however, could result from different sites or anisotropy of one site.

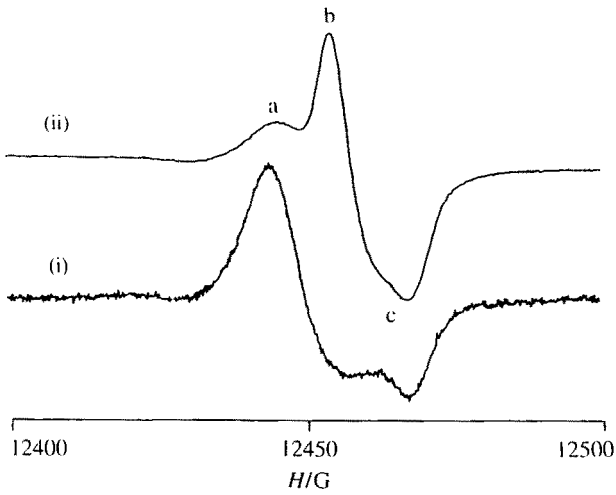

FIG. 2. Q-band EPR spectrum of (i) unrotted straw and (ii) pond-rotted straw. The Q-band EPR spectrum (35 GHz) shown was recorded on (i) unrotted straw (sample 1, Tables 1 and 3) and an air-dried sample of pond-rotted straw (sample 4, Tables 1 and 3). Microwave power 20 dB. The signals arising from the straw labeled a, b, and c are discussed in the text and g values provided in Table 3. A weak feature barely evident at the low-field side (approx. 1243 mT) of the radical signals is one of six hyperfine lines due to manganese.

DISCUSSION

We discuss here the presence of a persistent free radical in unrotted straw, decomposing straw, and in the macromolecular material recovered from the water in which straw was decomposing and suggest that our data indicate that the radical is associated with the lignin fraction of the straw. The role of microorganisms associated with rotting straw in the overall EPR signal is considered. Finally, we speculate on the significance of our findings to the observed selective antibiotic effects of water in which straw is rotting and to questions of water quality in general.

Our data extend the range of plant materials reported to contain stable radicals by showing the presence of a radical that gave a broad signal (X-band EPR) with a g value of 2.004 in an agricultural sample of barley straw. The major radical signal obtained on the same material with Q-band EPR spectroscopy had a g value of 2.0046. This value is consistent with the radical being of the semiquinone type, in which g values occur in the range 2.0038–2.0047

(Saiz-Jimenez and Shafizadeh, 1985). Whether the radical we detected in the unrotted straw is similar to that reported as being ubiquitous in senescent plant material (Hendry et al., 1994) has yet to be determined. The radical was detected at room temperature without recourse to grinding the sample, suggesting that it is inherently stable in natural straw and not a result of physical disruption that can induce a radical signal (Ludwig, 1971; Partidge et al., 1993).

Even when straw had undergone aquatic decomposition for up to six months, a radical persisted in association with the straw. Under such conditions microbial lignolytic activity was minimal, as evidenced by the intact appearance of the straw. Furthermore, alkali treatment of the unrotted straw, designed to solubilize lignin (Jackson, 1977), left the straw without an EPR signal. We suggest, therefore, that the radical we detected in straw is within the polyphenol lignin fraction, which typically comprises some 15% by weight of straw. The high syringyl content of barley straw lignin (Boon, 1989) may support the presence of radical sites in this material. Steelink (1966), for example, showed that syringyl (dimethoxy-substituted) lignin contained more free radicals than guaiacyl (monomethoxy) lignin. The X-band spectral data indicate that the radical of fresh straw was similar to that of the rotted straw. However, the additional information provided by Q-band spectra suggested that while the radical sites seen in fresh straw survive aquatic decomposition, an additional signal (b) was present in rotted straw.

Not only was a radical detected in rotted straw, but the material known to contain solubilized lignin components, which leached naturally into the surrounding water, also demonstrated a radical signal. The radical in the dried soluble lignin preparation was stable at room temperature for at least eight weeks. While X-band EPR spectroscopy indicated that the radical was indistinguishable from that of the rotted straw from which it was derived, Q-band spectra of the two samples indicated dissimilarities. Indeed, the EPR spectrum of the soluble lignin material was similar to that of the fresh straw prior to rotting, further indicating a polyphenolic origin for the radical population observed in the unrotted straw. The soluble lignin preparation did not show the additional signal demonstrated in the rotted straw samples. Whatever component accounted for the additional radical site in the straw following decomposition either: (1) did not solubilize into the surrounding water, (2) was unstable in solution, or (3) was not precipitated within the macromolecular lignin complex. Possibly, it remained in the ethanolic phase, which was discarded.

We suggest two possible explanations for the additional radical signal associated with rotted straw. First, the radical may result from the presence of fungal decomposers that can contain compounds (e.g., melanins) that have an indigenous population of stable free radicals. Our preliminary evidence from the X-band EPR spectra (data not shown) of mycelium of two pigmented fungi, a *Fusarium* species and *Alternaria alternata*, a dematiaceous hyphomycete, does

indeed support this suggestion. Further experiments in which natural isolates are grown under defined conditions are needed to produce samples for detailed spectroscopy. Species of *Fusarium* and black-pigmented fungi are common isolates from rotting straw (Pillinger et al., 1992). It is interesting that Peng et al. (1992) showed that the presence of a fungal contaminant of either *Fusarium* or *Alternaria* increased the free radical signal of grain substrate, but they did not consider that the increase may be due directly to the presence of fungal material. Secondly, while fungi may contribute radical signal b, it is also possible that this additional radical signal in rotted straw may be produced indirectly by fungal or other biological action on the lignin itself enhancing (Steelink, 1966) or generating a radical. Experiments are planned to distinguish between the possible provenances of the radical b.

The free radical signature of lignin is clearly remarkably stable, possibly protected in the polymeric matrix. However, the potential reactivity of the radical-containing lignin fraction when dissolved in water is unknown. While the radical site may remain protected even when the macromolecular material is in solution, it is possible that breakdown of the macromolecule will expose the radical site(s). Indeed Everall and Lees (1996) recently detected low-molecular-weight phenolic materials in water in the vicinity of submerged straw. The fate of the additional radical site associated with the rotted straw is also unknown.

It is now accepted that barley straw rotting in well-oxygenated water prevents algal growth. The use of straw is exploited widely to control nuisance algae (Ridge and Barrett, 1992) and cyanobacteria (Newman and Barrett, 1993) in fresh water, including potable supply reservoirs (Barrett et al., 1996). The oxidized polyphenolic content of straw has been implicated in the observed toxicity to algae (Pillinger et al., 1994), and syringyl lignin appears to be more toxic than guaiacyl lignin (Pillinger et al., 1995). Peng et al. (1992) postulated that a stable free radical dissolved in drinking water containing a high concentration of fulvic acids was one of the major causes of Kashin-Beck disease. We have yet to determine whether the observed toxicity of rotting straw towards algae can be attributed in any way to damage from radicals derived from straw polyphenolics.

CONCLUSIONS

We suggest the importance of our observations is that a radical was detected after recovery from aqueous solution and that the signal was associated with a material derived from naturally decomposing plant litter. An additional radical not observed in fresh straw was associated with the decomposing straw. Whether radicals associated with the lignin fraction of plant litter are available to effect damage to aquatic organisms or to organisms ingesting contaminated water has yet to be determined.

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PINE NEEDLE OIL CAUSES AVOIDANCE BEHAVIORS IN POCKET GOPHER *Geomys bursarius*

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Abstract—Essential oils from coniferous trees contain secondary metabolites that act as feeding deterrents for a number of herbivorous mammals. We investigated effects of pine needle oil on feeding and other behaviors of herbivorous plains pocket gophers. In experiment 1, pocket gophers were offered sweet potato from single feeding stations placed in home cages of individually housed animals. Stations contained either a scent dispenser with pine needle oil or mineral oil. Subjects removed significantly less food from stations scented with pine needle oil. Experiment 2 was performed to investigate neophobic responses to odors. *d*-Pulegone, presented under conditions identical to those used in experiment 1, did not reduce food removal compared to mineral oil. In experiment 3 pocket gophers were observed in a maze consisting of a start box connected to two goal boxes by tunnels. One goal was scented with pine needle oil, the second with mineral oil. Subjects entered goals scented with pine needle oil significantly less frequently than goals scented with mineral oil and spent less time there. They performed all recorded behaviors at lower frequencies while located in pine-scented goals. In experiment 4 animals were introduced into a maze consisting of a start box from which two soil-packed tunnels could be entered. Embedded in the soil of one tunnel was a barrier of electrical cable that had been soaked in pine needle oil, the second tunnel contained a barrier of cable soaked in mineral oil. Pocket gophers gnawed

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significantly less insulation from cable treated with pine needle oil than from cable treated with mineral oil. Our results show that constituents in pine needle oil are aversive to plains pocket gophers. Under natural conditions they may function as feeding deterrents. Some of the compounds may be suitable repellents for control of pocket gopher damage.

Key Words—Pocket gophers, avoidance behavior, feeding deterrents, plant secondary metabolites, pine needle oil.

INTRODUCTION

Plants produce a great variety of secondary metabolites such as phenolics, alkaloids, and terpenoids that act as defense against herbivores (Harborne, 1991; Haslam, 1988; Langenheim, 1994). Many of these compounds are toxic, and herbivorous mammals have evolved physiological mechanisms to protect themselves from this toxicity (Freeland and Janzen, 1974; McArthur et al., 1991). They also possess behavioral mechanisms to avoid ingestion of some plants or parts of plants, and these mechanisms often are based on chemosensory detection of secondary metabolites. Snowshoe hares (*Lepus americanus*), for example, are highly selective feeders. Their avoidance of buds and catkins of Alaskan green alder (*Alnus crispa*) is mediated by two phenolic compounds, pinosylvin and pinosylvin methyl ether (Bryant et al., 1983; Clausen et al., 1986). Mixtures of phenolic secondary compounds in some structures and growth stages of balsam popular (*Populus balsamifera*) cause avoidance by the same species (Reichardt et al., 1990a). Snowshoe hares also avoid winter apical birch (*Betula* spp.) twigs containing phenolic glycosides (Iason and Palo, 1991; Sunnerheim et al., 1988), juvenile growth stages of winter-dormant paper birch (*Betula resinifera*) bearing papyriferic acid (Reichardt et al., 1984), as well as food sources with germacrone, a terpene from Labrador tea (*Ledum groenlandicum*) (Reichardt et al., 1990b).

Essential oils from conifers also contain secondary metabolites, mostly terpenoids and phenols, that act as feeding deterrents for snowshoe hare (Sinclair et al., 1988), meadow vole (*Microtus pennsylvanicus*) (Roy and Bergeron, 1990), moose (*Alces alces*) (Sunnheim-Sjoberg, 1992; Sunnheim-Sjoberg and Hamalaien, 1992), red deer (*Cervus elaphus*) (Elliott and Loudon, 1987), and mule deer (*Odocoileus hemionus columbianus*) (Schwartz et al., 1980). Industrial pine oil, a by-product of the wood pulp industry, has shown some promise as a feeding deterrent for snowshoe hare and Townsend's vole (*Microtus townsendii*) (Bell and Harestad, 1987).

Responses of pocket gophers to secondary plant metabolites have not been studied in any detail. For *Thomomys*, however, Radwan et al. (1982) showed that ponderosa pine (*Pinus ponderosa*) seedlings from some geographic areas

are protected against attack, while other seedlings are heavily damaged. Different parts of the same seedling are also discriminated during feeding. Preferences are negatively correlated to stem oils, indicating that some terpenoid constituents of conifer oils are able to protect trees from gopher attacks, and therefore are potential feeding repellents.

In the present study the effects of pine needle oil and of *d*-pulegone on feeding and other behaviors of plains pocket gophers (*Geomys bursarius*) were assessed. Pocket gophers are generalist herbivores that forage mostly underground by excavating tunnels to reach food plants. These rodents cause problems by gnawing through underground cable they encounter while digging. Moreover, they are serious pests in agricultural and reforestation areas (Chase et al., 1982). Species of the genus *Thomomys*, in particular, hinder conifer reforestation by clipping roots and girdling trunks (Burton and Black, 1978; Gottfried and Patton, 1984; Radwan et al., 1982) and are also a persistent problem in orchards (Sullivan et al., 1987). Plains pocket gophers inhibit establishment of red oak (*Quercus rubra*) and white pine (*Pinus strobus*) (Huntly and Inouye, 1988) and can damage Christmas tree plantations (Hegdal, personal communication).

METHODS AND MATERIALS

Subjects. Twenty-four adult plains pocket gophers, 15 females and 9 males, served as subjects. Eighteen had been trapped near San Antonio, Texas, and six were born in the laboratory to females that were pregnant when caught. All animals were individually housed in stainless steel cages (60 × 50 cm, 22 cm high), containing aspen chips, pieces of plastic pipe (15 cm long, inner diameter 8 cm), and cardboard boxes for nesting. Purina Guinea Pig Chow, Mazur Omnivore A pellets, and a mixture of rolled oats, sunflower seeds, and peanuts were fed ad libitum and were available during all experiments. Dandelion, kale, and crab apple branches were given occasionally. The animal room was kept at approximately 21°C; light and dark phases were adjusted to reflect seasonal changes in hours of day light in Philadelphia (14L:10D in July; 10L:14D in December).

Stimulus Material. Pine needle oil was purchased from the Penn Herb Co., Philadelphia, Pennsylvania (Siberian Pine Needle Oil, catalog no. 549). A partial analysis of the oil, performed in our laboratory, showed α-pinene, β-pinene, and myrcine to be among its constituents (Wager-Pagé et al., 1996). *d*-Pulegone was obtained from International Flavors and Fragrances. Light white mineral oil NF (Lannett Co., Philadelphia, Pennsylvania) served as a control stimulus.

Experiment 1. The effect of pine needle oil on retrieval and consumption of preferred food was assessed in single-choice tests. Ten wild-born and six captive-born animals served as subjects. During each test a scented feeding

station containing 10 g of diced sweet potato was placed into the subject's home cage for 1 hr. The amount of sweet potato remaining in the feeding station at the end of the trial was used as a measure of pine needle oil repellency.

Each feeding station consisted of a plastic tunnel (25 cm long, 8 cm ID) that was sealed at one end by inserting a 6-cm-deep stainless steep cup. The cup contained the sweet potato and a scent dispenser attached to the cup above the food. Tunnels were placed parallel to the front walls of the home cages and were kept from rolling by securing them with a clean brick.

Stimulus fluids (100 μl) were applied to filter paper, 3 × 9 cm, folded into 3-cm^2 pads. Papers were placed into plastic mesh capsules (HistoPrep, Fisher Scientific; 25 × 6 mm). These scent dispensers allowed the animals to smell but not to contact the stimuli and prevented contamination of the food with odorants. The volume of 100 μl resulted in a moderately strong scent that could be detected easily by the human observer at the end of the trials.

Different sets of tunnels and cups were used with mineral and pine needle oil to avoid cross-contamination. All testing equipment was machine washed at 82°C and thoroughly rinsed with clean water between trials. Scent dispensers were discarded after each trial.

Subjects were tested once with each stimulus on different days. Only eight individuals per day were tested, four receiving pine needle oil and four receiving mineral oil. The order of stimulus presentation was counterbalanced across subjects. The experiment was conducted during November. All trials were given between 14:00 and 16:00 hr. This time lies within the species' activity period under natural conditions (Benedix, 1994).

Results were evaluated in a two-factor analysis of variance (ANOVA). The independent factor was groups (two levels: wild-caught and laboratory-born individuals). The dependent factor was stimulus type (two levels: pine needle oil and mineral oil). Tukey post-hoc tests (Winer, 1962) were used to isolate differences among mean grams of food taken.

Experiment 2. To assess the possibility that responses to pine needle oil were the result of neophobia, responses to a novel odor were tested in eight wild-caught pocket gophers that had never experienced the odor and that were not included in any of the experiments with pine needle oil. *d*-Pulegone, a bird repellent (Mason, 1990) with a potent mint scent (for humans) was used. Mineral oil served as a control. The experiment was performed in July. Testing procedures were identical to those used in experiment 1. Results were evaluated by paired *t* test.

Experiment 3. The effects of pine needle oil on the behavior of pocket gophers in a maze apparatus were investigated using the 16 animals tested in experiment 1. The maze consisted of an opaque plastic start box (30 × 20 cm, 14 cm high) covered with a clear Plexiglas lid. The start box was connected to two clear plastic goal boxes (28 × 28 cm, 12 cm high) via tunnels. Each tunnel

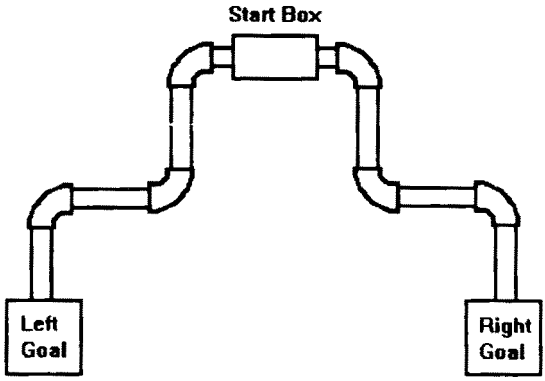

FIG. 1. The maze used in experiment 3.

consisted of three 25-cm-long secretions of translucent PVC tubing (5 cm ID) connected with 90° elbows (Figure 1). The start box was ventilated by connecting a hose attached to an opening in the lid of the box to the air exhaust of the test room.

Each goal box contained 200 ml of fresh aspen bedding and an aluminum plate (5 cm wide, 10 cm long, 4 mm thick). A HistoPrep scent dispenser containing a 3 × 9-cm strip of folded filter paper was taped to each plate to prevent the subjects from removing the dispensers from the goal box. Three cubes (2 × 2 × 2 cm) of sweet potato were placed on each plate in front of the scent dispenser.

To habituate the animals to the maze and to test for the presence of side preferences, each subject was given one trial in the clean maze, with both goal boxes containing empty scent dispensers. Following this trial, each gopher was given two trials in which the dispenser in one goal box contained 100 μl pine needle oil and the dispenser in the second goal box 100 μl mineral oil. To avoid cross-contamination, one goal box was dedicated to use with pine needle oil, the other with mineral oil. Their left–right positions were counterbalanced across subjects and replications. Between trials, equipment was washed with detergent, rinsed in water, and then soaked in 70% ethanol for several minutes.

Trials were 10 min long, divided into 60 intervals of 10 sec, indicated by an audible timer signal. At each 10-sec signal, an observer noted the location of the animal as being in the start box, the left or right tunnel system, or the left or right goal box. Behaviors were scored as Hansen frequencies (Altmann and Wagner, 1970) per 10-sec interval. For each interval the subject received a score of 1 for each of the behaviors defined below, if this pattern was displayed. The following behaviors were recorded as Hansen frequencies: (1) enter

left/right goal box, (2) dig in the litter of the left/right goal box, (3) eat in left/right goal box, (4) carry food from left/right goal box, and (5) groom self in left/right goal box.

Subjects were tested between 14:00 and 16:00 hr and were only removed from their home cages when they were active. Gophers were transferred to the maze in a plastic tube and released into the start box. The 10-min trial began with the first 10-sec interval after the subject left the start box. The experiment was conducted between December and March.

For results obtained during habituation trials, differences between the subjects' location in the left and right side of the maze and between behaviors performed in these locations were compared by paired *t* test. Paired *t* tests were also used to analyze location and behavior scores recorded during experimental trials.

Experiment 4. The effects of pine needle oil on cable gnawing were investigated. Trials were performed in a maze where subjects encountered electrical cable embedded in soil. The weight of material removed from the cable in the course of an 18-hr trial was taken as a measure of the effectiveness of the pine needle oil as a repellent in this context.

The maze consisted of a linear arrangement of a start box (60 × 40 cm, 40 cm high) from which two 45-cm-long tunnels (15 cm ID) were accessible. Each tunnel was sealed at one end and completely filled with soil. It contained a barrier of three electrical cables, set in a frame and embedded in the soil 35 cm from the tunnel entry. Copper conductor cable with PVC insulation and jacket (12 mm wide, 7 mm thick) was used. The frame was made from plastic pipe (10 cm long, 12 cm ID) that fitted inside the tunnel. Three 14-cm-long pieces of cable were inserted into holes in the pipe and secured by bending them on the outside of the frame. The wires crossed each other and formed a netlike barrier that blocked the gopher's progress as it dug along the tunnel.

Twelve of the animals used in experiments 1 and 3 were each given four trials in the maze. During the first two trials, cables were untreated. During the third and fourth trials, one tunnel contained cables that had been immersed in pine needle oil for six days. The second contained cables that had soaked in mineral oil for six days. During trials with scented cable, one of the tunnels and frames was used only with pine needle oil, the other only with mineral oil. The left–right positions of the tunnels containing mineral and pine needle oils were counterbalanced across subjects and replications.

Trials were conducted overnight. Prior to each trial, the electrical cable was weighed, inserted into the frames, and embedded in fresh, commercially obtained top soil. Soaked cables were completely dried before weighing. The start box was supplied with standard diet and a small apple.

Each subject was removed from its home cage at 16:00 hr and placed into the start box of the maze. At 10:00 hr the following day, the animal was returned

to its home cage. The cables were then weighed and discarded together with the used soil. Between trials, equipment was washed in a commercial cage washer at 82°C and thoroughly rinsed with clean water. Experiment 4 was performed in July and August.

Differences among weights of the material removed from the cables under unscented and scented conditions were analyzed in a two-factor ANOVA for repeated measures. Factors were conditions (two levels: clean maze and scented maze) and stimulus type (two levels: mineral oil and pine needle oil). For the latter factor, gnawing of mineral-oil-soaked wire was compared with gnawing of wire soaked in pine oil. Tukey post-hoc tests (Winer, 1962) were used to isolate differences among means.

RESULTS

Experiment 1. ANOVA revealed a significant difference between stimulus types ($F = 72.9$; 1,13 *df*; $P < 0.00001$) but no interaction between groups and stimulus types. Tukey tests showed that wild-caught as well as laboratory-born pocket gophers retrieved much less food from stations scented with pine needle oil than from stations scented with mineral oil (Figure 2).

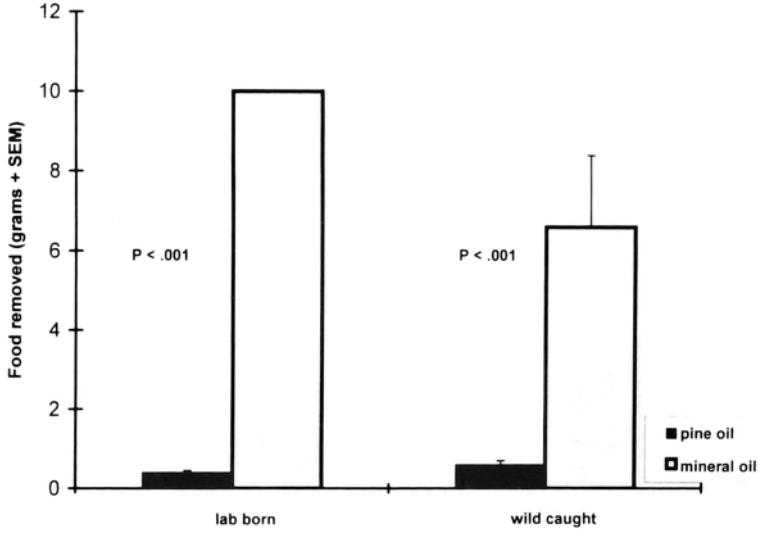

FIG. 2. Mean amount of sweet potato (SEM) removed by laboratory and wild born pocket gophers in experiment 1 from feeding stations scented with pine needle oil or mineral oil.

Experiment 2. Pulegone had no effect on food retrieval. The animals removed an average of 3.1 g (SEM 1.1 and 1.2, respectively) of food from each feeding station.

Experiment 3. The pocket gophers did not exhibit locational or behavioral side preferences during trials in the unscented maze. However, for experimental trials, *t* tests revealed significant differences between location scores in the pine needle oil scented and mineral oil scented parts of the maze and between scores for all behaviors performed in these locations (Figures 3 and 4). There were no differences in the scores obtained for being located in any of the three tunnels leading to the pine and mineral oil scented goal boxes. However, subjects entered pine needle oil scented goals significantly less frequently than mineral oil scented goals, and they were less frequently located there.

All behaviors were performed at significantly lower levels in the goal containing pine needle oil (Figure 4). Eating was particularly strongly affected, with only two of the 16 animals, one male and one female, eating while inside the pine needle oil scented goal, as compared to 12 of 16 animals eating while inside the control goal.

Experiment 4. ANOVA revealed a significant difference between stimulus types ($F = 8.0$; 1,11 *df*; $P < 0.016$) and a significant interaction between conditions and stimulus types ($F = 8.8$; 1,11 *df*; $P < 0.012$). The analysis was

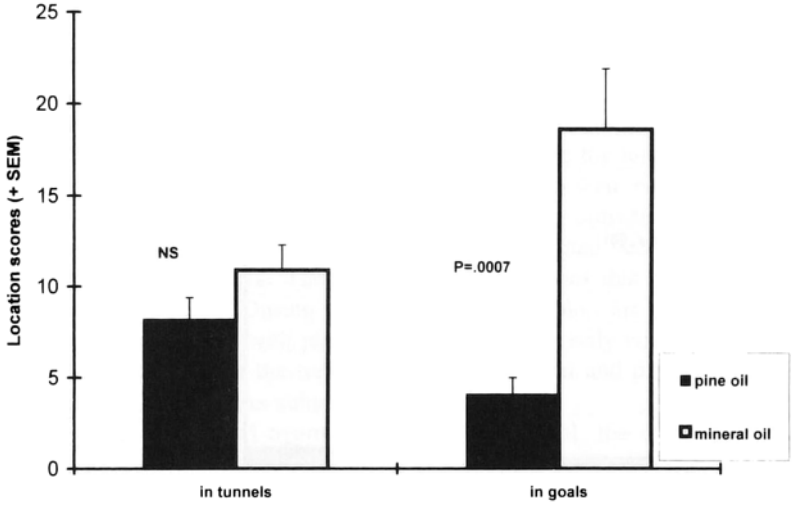

FIG. 3. Mean (+SEM) scores obtained by pocket gophers in experiment 3 for locations in tunnels leading to each goal box and in goal boxes scented with pine needle or mineral oil. NS, no significant difference.

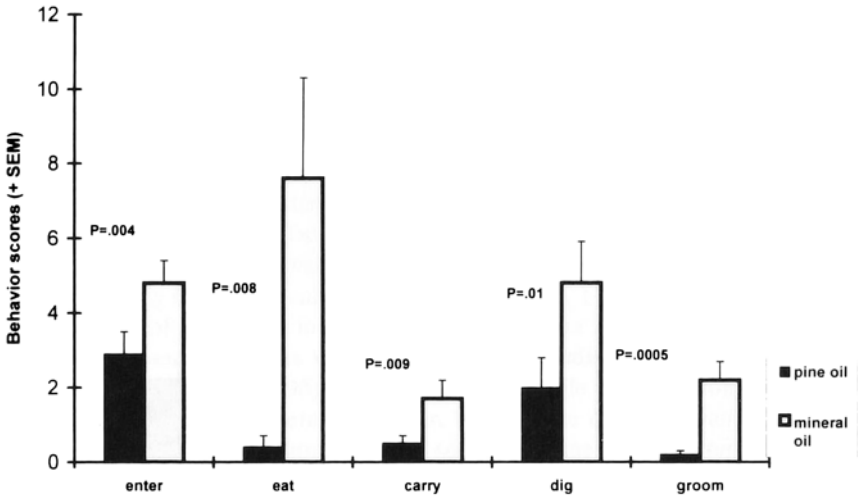

FIG. 4. Mean (+SEM) behavior scores obtained by pocket gophers in experiment 3 for entering goal boxes scented with pine needle or mineral oil and for digging, eating, carrying food, and grooming in these locations.

interpreted in terms of the interaction. During habituation trials with unscented electrical cable, no side preferences were displayed. About equal amounts of material were removed from the cables in each tunnel (Figure 5). Impregnating cable in one of the tunnels with pine needle oil did not reduce the overall amount of material removed from both tunnels, but shifted gnawing activity from pine needle oil impregnated material to that impregnated with mineral oil. Tukey tests showed that subjects removed significantly less material from wire scented with pine needle oil than that scented with mineral oil or from clean wire (Figure 5).

DISCUSSION

For the plains pocket gopher, pine needle oil is an aversive stimulus that influences behavior in a variety of contexts. Food caching was significantly reduced, as was consumption. Pine needle oil also caused area avoidance. In the maze, pocket gophers entered pine-scented chambers less frequently than chambers scented with mineral oil. All behaviors were performed less frequently in the presence of pine scent, perhaps as a result of spending less time there. Similar results were obtained in a more recent study with northern pocket gophers (*Thomomys talpoides*) that were tested in a maze comparable to that used in

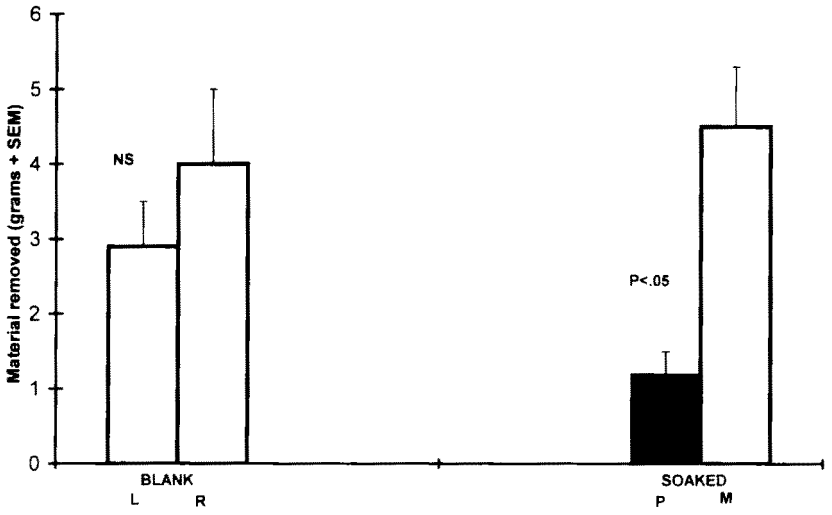

FIG. 5. Mean (+SEM) weight of material removed in experiment 4 from clean cable and from cable soaked in pine needle oil or mineral oil.

experiment 3 (Epple et al., 1996). In experiment 4, pine needle oil significantly reduced damage to soil embedded cable.

In the present study, individuals born in the laboratory and never before exposed to pine needle oil responded as strongly as wild-caught animals, about whose previous experiences with this oil or any of its components we have no information. Thus, it appears that one or several constituents of the pine oil are innately aversive to plains pocket gophers. The indifference of the animals to pulegone, a compound that is very odoriferous for the human nose, shows that this aversion is not based on neophobia. Moreover, there was no habituation to pine needle oil across experiments involving the same subjects.

In experiment 4, the pocket gophers contacted the pine needle oil soaked cable during chewing, and the stimulus could have influenced behavior via olfaction, the trigeminal system, which mediates irritation, other nasal chemosensory systems, or taste. In experiments 1 and 3, however, stimuli were enclosed in plastic capsules, and the animals could only perceive volatile cues. Although nasal trigeminal chemoreception could have been involved, the animals showed no behavioral indication of irritation, and it appears likely that avoidance was mediated, at least in part, by olfaction. Olfaction also appeared to be involved in mediating responses of northern pocket gophers to pine needle oil (Epple et al., 1996). Secondary plant metabolites are feeding deterrents for several mammalian herbivores (Iason and Palo, 1991; Langenheim, 1994; Reichardt et al.,

1984, 1990a,b; Roy and Bergeron, 1990; Sinclair et al., 1988; Sunnerheim-Sjoberg and Hamalainen, 1992). Some of these species avoid deterrents on the basis of olfactory cues alone. Snowshoe hares and Townsend's voles avoid feeding from sources associated with the scent of pine oil extracted from pulp waste (Bell and Harestad, 1987). Snowshoe hares also avoid feeding on conifer seedlings to which odor dispensers with pinosylvin, a phenolic compound from Alaskan green alder (Clausen et al., 1986), have been attached (Sullivan et al., 1992). The odors of crushed foliage from lodgepole pine (*Pinus contorta*) and Sitka spruce (*Picea sitchensis*) and of some of their monoterpene constituents inhibit feeding in red deer calves (Elliott and Loudon, 1987). Mule deer respond to the odors of juniper oil in selecting food (Schwartz et al., 1980).

The present results as well as those of our studies with northern pocket gophers (Epple et al., 1996) suggest that pine needle oil is a strong repellent for pocket gophers in general. However, the possible role the essential oil and its constituents play in controlling food selection in these species remains to be elucidated. It is likely that the animals responded to the pine needle oil because it contains compounds that act as feeding deterrents under natural conditions. Grasses constitute the most important part of the yearly diet of plains pocket gophers, although succulent forbs are preferred in spring and summer (Behrend and Tester, 1988; Benedix, 1993; Luce et al., 1980; Myers and Vaughan, 1964). Grasses and forbs do not contain essential oils, and grazers have less well-developed detoxification mechanisms than browsers and may depend more on avoidance (McArthur et al., 1991). However, plains pocket gophers consume roots of white pine and red oak (Huntly and Inouye, 1988), and under snow cover also eat above-ground parts of pine trees in Christmas tree plantations (Hegdal, personal communication). Northern pocker gophers contribute heavily to seedling mortality in fir plantations (Gottfried and Patton, 1984; Radwan et al., 1982). It is likely, therefore, that both species encounter essential oils from conifers during foraging.

Essential oils from conifers are chemically complex, being predominantly composed of monoterpene hydrocarbons (Langenheim, 1994; Radwan et al., 1982). There are differences in the composition of oils among different conifer species, different populations of the same species, individual trees, and different parts of the same plant, and animals respond to such differences (Langenheim, 1994). In the only study on pocket gophers known to us, feeding preferences of *Thomomys* (species not given) for ponderosa pine seedlings were negatively correlated to the β-pinene plus sabinene component of stem oils but positively correlated to the total terpene yield of root oils. Leaf oils, on the other hand, showed no correlation with feeding preference (Radwan et al., 1982).

In conclusion, the strong avoidance of odors from the pine needle oil used in this study suggests that compounds from this essential oil are ecologically significant for *Geomys bursarius* and might make very promising feeding repel-

lents for other species of pocket gophers as well. Moreover, the use of pine needle oil constituents in reducing gnawing damage to underground cable should be considered. However, studies on isolation and identification of active compounds from the brand of oil used in the present experiments are needed before its use as a repellent can be explored.

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CHEMIOTROPIC BEHAVIOR OF FEMALE OLIVE FLY (*Bactrocera oleae* GMEL.) ON *Olea europaea* L

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Abstract—An interpretation is given of a number of observations on the chemiotropic behavior of *Bactrocera oleae* in connection with olive maceration water and the fly's return to the olive groves after the first summer rains. To this end, the headspace of both maceration water and leaf leaching water, simulating rainfall, were examined. In both cases, the presence of ammonia, which is generally known to attract fruit flies (Diptera, Tephritidae), was detected and, for the first time, in addition to other compounds that are inert for the fly, the presence of styrene was also detected. This aromatic hydrocarbon was found to be a strong attractant. It is shown that both ammonia and styrene are products of the metabolism of microbial flora present on the olive and leaf surface.

Key Words—*Olea europaea*, attractants, *Bactrocera oleae*, Diptera, Tephritidae, olive maceration water, olive leaf leachate, microbial metabolites, styrene, ammonia.

INTRODUCTION

Despite the gradual increase in our knowledge of the complex relation between the olive fly, *Bactrocera oleae* (B. oleae), and the host plant (*Olea europaea*) the causes of the following chemiotropic behaviors are still unknown: (1) drowning of numerous females in the maceration water during the process of sweet-

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ening olives for food purposes (Vita and Cirio, 1980); and (2) large-scale return of the fly to olive groves after the first abundant summer rains (Girolami et al., 1982). It is reasonable to postulate the presence of attractants in both the maceration water and on rain-soaked leaves, particularly in warm weather. As part of our on-going work on the interaction between *B. oleae* and the olive tree (Scarpati et al., 1993; Lo Scalzo et al., 1994), we report here the results of research leading to the identification of chemical substances responsible for attraction of flies to olive maceration water and to rain-soaked leaves.

METHODS AND MATERIALS

General Experimental Materials

Olive leaves or olives (Frantoio or Leccino cultivars) were collected from July to November in the Lazio region of Italy. Ammonia was determined by the colorimetric test Merckoquant (Merck, Cod. 10024). Aldrich GC purity samples were used for laboratory bioassays. The systems used for the analysis of the volatile compounds were described in previous publications (Scarpati et al., 1993; Lo Scalzo et al., 1994).

Preparation of Olive Maceration Water for Determination of Attractancy, Headspace Volatiles, and Ammonia

One kilogram of olives was soaked in 1 liter of H_2O and allowed to stand at room temperature for 8–10 days. The decanted maceration water solution (MW) was used for the reported determinations. The same procedure was used for the preparation of maceration water in which microbial growth was inhibited by adding 1 g of streptomycin per liter of H_2O (MWS).

Preparation of Leaf Leachate Water for Determination of Attractancy and Ammonia Release

One kilogram of freshly cut olive branches was placed in a stoppered vessel and shaken constantly for 15 min with 200 ml of H_2O. The decanted leaf leachate solution (LW) was used for the reported determinations. The same procedure was used to produce leaf leachate water in which microbial growth was inhibited by adding 200 mg of streptomycin (LWS).

Preparation in the Field of Enriched Leaf Leachate Water for Headspace Volatile Analysis

One liter of distilled water was sprayed for about 5 min on a leafy branch about 1 m long that had not been cut from the tree. The water dripping off the

branch, simulating rainfall, was collected in a large vessel placed underneath, and then it was sprayed on the same branch twice more. The same solution was used repeatedly on nine different branches, thus allowing about 0.5 liter of enriched leachate water to be obtained.

Chemical Investigations

The headspace volatiles from MW, MWS, and enriched leaf leachate water were trapped and analyzed with the following methods described by Scarpati et al. (1993). Water solutions were stripped with pure nitrogen for 15–120 min at a flow rate of 50 ml/min. Adsorption of the volatiles at the exit of the container was performed with a Supelco Carbotrap 300, 115 mm × 4 mm ID. All connections were made with steel–glass or glass–glass contacts secured with Teflon pipes. The adsorbed components were desorbed by heating the Carbotrap with a Supelco Thermal Desorption Unit (TDU, model 850) under a helium flow and analyzed with a GC-MS (HP 5890/HP 5971 A) system linked with a data processor (HP 59970 MS Chemstation). The GC column was a capillary Supelco SPB-1 (1% methyl-phenylsilicone), 30 m × 0.2 mm ID, film thickness 0.2 μm.

In order to identify and determine the less volatile compounds, such as 2-phenylethanol, which were not well represented in the headsapce volatiles, 500 ml of MW were partioned for 8 hr with a *n*-pentane–methylene chloride solution 6:4, in a continuous liquid–liquid extractor. The final MW extract in *n*-pentane–methylene chloride was concentrated to a small volume through a Vigreux column for GC analysis.

Ammonia determinations were carried out on MW, MWS, LW, and LWS, after a paper filtration with the colorimetric method (Merckoquant, Cod. 10024).

Laboratory Bioassays

Bioassays described by Scarpati et al. (1993), were conducted under laboratory conditions of 21–25°C and 40–50% relative humidity. Adult olive fruit flies from groves in the Lazio region were transferred to Plexiglas cages (20 × 20 × 20 cm) and fed a diet consisting of a mixture of sugar (33%), soy flour (33%), and yeast hydrolysate (33%). A rearing procedure was adopted using fruits of the Itrana olive cultivars, available until April. The insects were transferred to cages 10 days after eclosion and subjected to the tests, during which water was constantly available but food was removed in order to avoid interference.

Two different bioassays were done in order to ascertain the effect of the sample on the behavior of gravid females of *B. oleae*.

Attractancy. In a Plexiglas cage (20 × 20 × 20 cm) containing gravid females (25 individuals), two 5-cm-ID Petri dishes were placed as far apart as possible. They contained 15 ml of test solution of a pure chemical or water

(blank). Every 30 min positions of the dishes were switched to eliminate position effects. An insect visiting a container was trapped in the dish containing test solution or water. Testing was carried out before sunset for 150 min, and the trapped individuals were counted.

Oviposition. The oviposition test, conducted in a Plexiglas cage (20 × 20 × 20 cm), was based upon the ability of three gravid females to oviposit on six natural fruits resting at the top of six test tubes (6 cm × 1.2 cm ID), three of which contained water (blank) and three filled to within 2.5 cm of the rim with test solution. An olive was placed on the rim of each test tube in such a way that volatiles evaporating from the solution came into contact with the fly (Figure 1). Six hours later, before sunset, oviposition punctures were counted, and the ratio between the total number of oviposition punctures on the three olives exposed to the test sample and the three exposed only to water was determined. The response index was defined as the mean value of the ratios resulting from ten bioassays.

Tests of attractancy and oviposition were conducted on MW, WMS, LW, LWS, and single synthetic chemicals identified in the headspace volatiles from olive MW, and/or leaf-enriched leachate water. All the pure chemicals used for

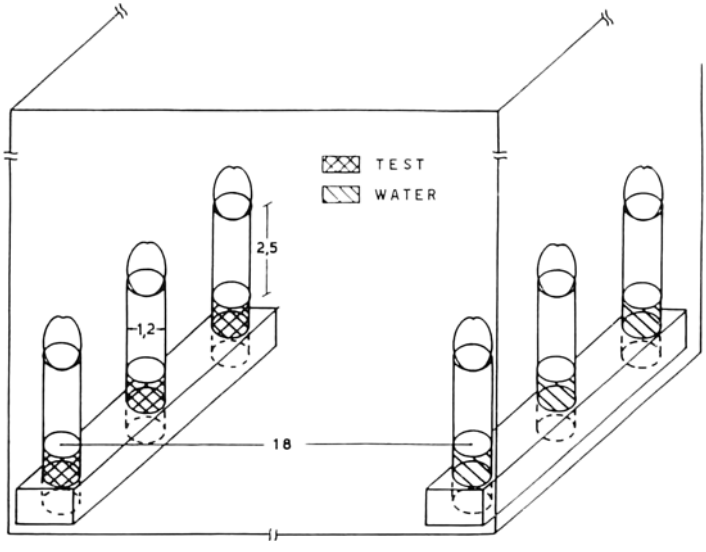


FIG. 1. Setup used in oviposition test with three gravid females and six olives; three test tubes contain chemicals and three water. All sizes are expressed in centimeters. Results are reported in Figure 4.

bioassays were bought from Aldrich (GC purity). They were dissolved or suspended in distilled water at a concentration of 5–20 ppm.

RESULTS AND DISCUSSION

Chemical Investigations. All the compounds identified are reported in Table 1. From MW headspace volatiles we identified the following, in order of decreasing GC peak area: styrene, **1**; *n*-pentanol, **2**, ethyl acetate, **3**; nonanal, **4**; 2-phenyl-ethanol, **5**; and, in smaller amounts, limonene, **6**; α-pinene, **7**; and β-pinene, **8**. Compounds **4**, **6**, **7**, and **8** had already been identified by us in the headspace volatiles from olives themselves. A stripping time of 15 min was sufficent to collect all the styrene because of its high volatility, but complete stripping of the other components required 2 hr.

Gas-chromatographic examination of the MW extract with methylene chloride–pentane indicated that 2-phenyl-ethanol is present in quantities four times greater than styrene. Styrene was not detectable in the headspace volatiles of MWS.

Compounds **1**, **5**, and **4**, in decreasing order, and small amounts of **6**; **7**; *p*-cimene, **9**; and *n*-octane, **10** were found in the headspace of the leaf-enriched leachate water. Compounds **6**, **7**, **9**, and **10** had already been identified by us in headspace volatiles from leaves themselves.

Ammonia is a generic attractant for many insects (Bateman and Morton, 1981) and is present in MW at concentrations of 80–150 ppm. Its content

TABLE 1. HEADSPACE VOLATILES

Compound	MW[a]	MW extract[a]	MWS[a]	Enriched leaf leachate[a]
1 Styrene	+++	+	–	+++
2 *n*-Pentanol	++	–	–	–
3 Ethyl acetate	++	–	–	–
4 Nonanal	+	sa[b]	sa	+
5 2-Phenylethanol	+	++++	sa	++
6 Limonene	sa	sa	sa	sa
7 α-Pinene	sa	sa	sa	sa
8 β-Pinene	sa	–	–	–
9 *p*-Cymene	–	–	–	sa
10 *n*-Octane	–	–	–	sa

[a]Relative areas of GC peaks are evaluated for each substrate.
[b]Small amounts.

decreases to 10–15 ppm in MWS, suggesting that it is largely produced from microbial fermentation.

Ammonia determination in LW prepared at different time intervals after the last rainfall indicates that values are fairly constant before rainfall (20–25 ppm), decrease immediately after rainfall, and then reach a maximum about 6–10 days later (55–60 ppm). The presence of streptomycin inhibits NH_3 formation also in LWS.

Attractancy Test. The MW was highly attractive (Scarpati et al., 1993), and MW was thus adopted as a positive control in the bioassays. The present results confirmed again the attractancy of MW. On dilution of MW with H_2O, the activity decreased, and the lower threshold occurred at a dilution of 1:4. MWS was completely devoid of activity. In order to demonstrate that the cancellation of activity was not due to a repulsive effect related to the addition of streptomycin, the attractancy of MW diluted with MWS 1:1 was compared with the attractancy of MW diluted with H_2O 1:1, and very similar responses were obtained. LW also was active, while LWS proved to be inactive. These results are shown in Figure 2.

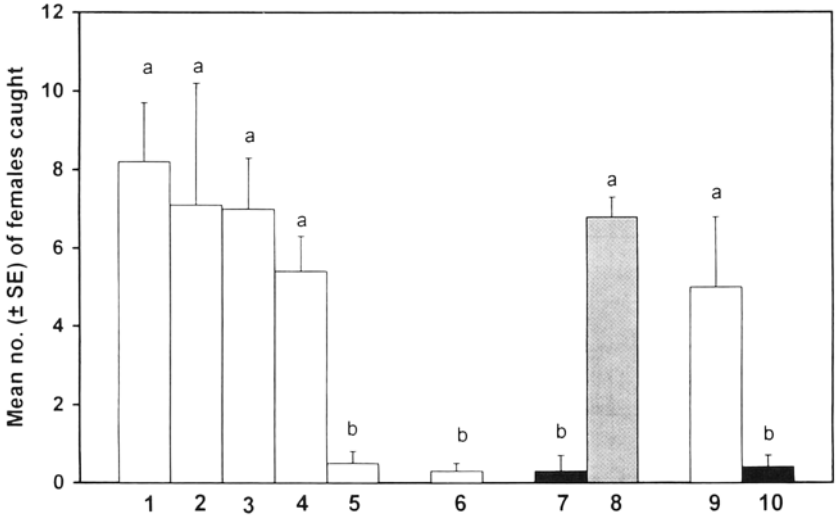


FIG. 2. Attractiveness to *B. oleae* females of MW, MWS, LW, and LWS in an olfactory attractancy bioassay. Replicate numbers are given in parentheses. Error bars represent standard error. Significant differences ($P < 0.05$) within each graph are indicated by different lowercase letters, as indicated by Duncan's (1955) multiple range test. **1,** absolute MW (30); **2,** MW–H_2O 1:1 (10); **3,** MW–H_2O 1:2 (10); **4,** MW–H_2O 1:3 (10); **5,** MW–H_2O 1:4 (10); **6,** H_2O (50); **7,** MWS (8); **8,** MW–MWS 1:1 (9); **9,** LW (8); **10,** LWS (8).

The single pure compounds identified in the headspace volatiles from MW and enriched leaf leachate were bioassayed with female *B. oleae*. Only styrene was highly attractive, while **2, 3,** and **4,** and its probable metabolic precursor, **5,** were inactive. Compounds **6–10** had been found previously to be inactive or weakly active. Attractancy peaked in water mixtures containing 10–15 ppm styrene, while it decreased strongly in mixtures containing 5 or 20 ppm styrene. Attractancy of styrene to *B. oleae* males was tested at concentrations of 10 and 15 ppm, and it was found to be practically inactive. Figure 3 gives a comparison of the activities of styrene, ethylbenzene, and toluene, which had shown significant activity in previous research (Scarpati et al., 1993).

Oviposition Test. All results of the oviposition test are shown in Figure 4. Styrene proved to be highly active in the oviposition assay, although it was not possible to ascertain whether the increased oviposition was due solely to a true stimulus of oviposition or only to olfactory attractancy (Figure 3). The other components from MW headspace, i.e., **2, 3, 4,** and **5,** were inactive in this test.

α-Pinene (**7**) had previously been found to act as a specific oviposition stimulus, but it proved only weakly active in the attractancy test. The comparison between **7** and **1** in the oviposition test showed that styrene is much more

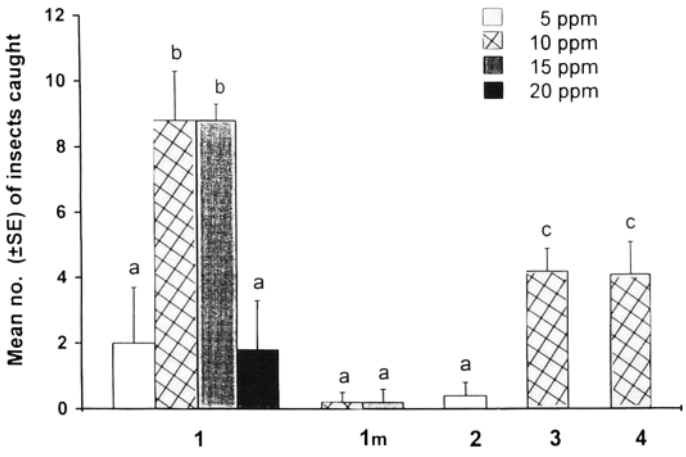


FIG. 3. Attractiveness to *B. oleae* females of styrene, toluene, and ethylbenzene in an olfactory attractancy bioassay at reported concentration in H_2O. Only styrene was assayed on males. Replicate numbers are given in parentheses. Error bars represent standard error. Significant differences ($P < 0.05$) within each graph are indicated by different lowercase letters, as indicated by Duncan's (1955) multiple range test. **1,** styrene on females (10); **1m,** styrene on males (10); **2,** H_2O (30); **3,** ethylbenzene (10); **4,** toluene (10).

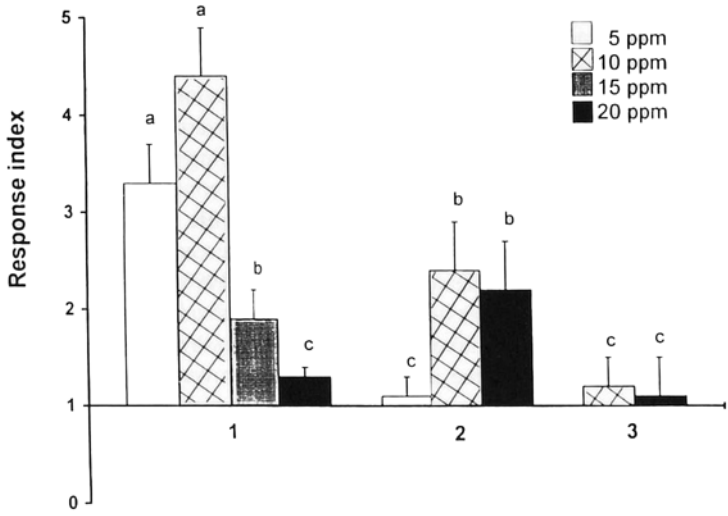


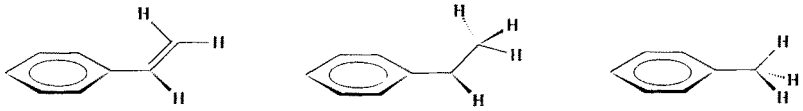
FIG. 4. The ratio between the total number of ovipositions of *B. oleae* on three olives exposed with the test sample and three exposed only with water was determined for each bioassay. The response index was defined as the mean value of the ratios from 10 bioassays. Replicate numbers are given in parentheses. Error bars represent standard error. Significant differences ($P < 0.05$) within each graph are indicated by different lowercase letters, as indicated by Duncan's (1955) multiple range test. **1,** styrene (10); **2,** α-pinene (10); **3,** ethylbenzene (10).

active than α-pinene at a concentration of 5 ppm, while the gap narrows at a concentration of 10 ppm. At 20 ppm, the activity of styrene decreases, while that of α-pinene remains constant.

CONCLUSIONS

Styrene, identified in MW and in leaf-enriched leachate was unexpectedly found to be highly attractive to the *B. oleae* female. Styrene is accompanied by 2-phenyl-ethanol, which was inactive and is likely the precursor of styrene.

Previously (Scarpati et al., 1993) we found that several components of the headspace volatiles from leaves and olives, i.e., ethylbenzene and toluene, exerted significant olfactory attractancy. Styrene has a structure related to such compounds, but conjugation with the aromatic ring of the vinyl group, which flattens the molecule, may produce a better steric interaction with the insect's receptor (Figure 5) (Metcalf, 1990).



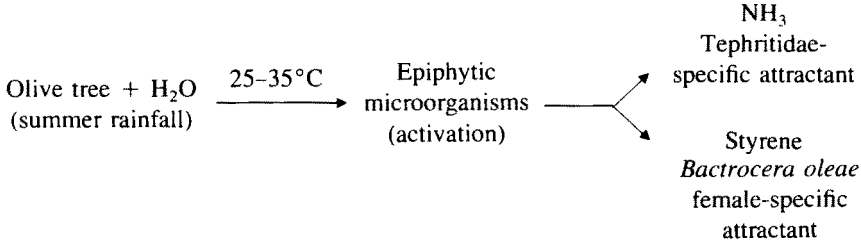
1 **ethylbenzene** **toluene**

FIG. 5. Tridimensional structures of styrene **1**, ethylbenzene, and toluene.

We have never found **1** or **5** in headspace volatiles from whole olives or leaves themselves. It is likely that, like ammonia, these compounds do not come from the plant's metabolism but from epiphytic microrganisms. These microrganisms have adapted to the surface of *Olea europaea* fruits and leaves (Ercolani, 1991), which are protected by a wax layer of triterpenic acids. The microrganisms are activated by the water (Richardson and Dowding, 1988), especially at relatively high summer temperatures. When microbial activity was inhibited by the addition of streptomycin to MW or LW, styrene was absent and ammonia was reduced to a minimum.

Both styrene and 2-phenyl-ethanol may be produced from the same intermediate, phenylacetaldehyde, a known breakdown product of phenylalanine. Styrene can also be a product of decarboxylation of cinnamic acid (Manitto, 1981).

The return of flies to the olive groves after the first summer rain may, in our opinion, occur according to the following pattern:



It is clear that in the coevolution of *B. oleae* with its host plant, the microbial populations living in symbiosis with the plant played an important role. The identification of a strong female attractant, styrene, together with the previous discovery of a strong repellent, (*E*)-2-hexenal (Scarpati et al., 1993; Lo Scalzo et al., 1994), provide a promising base for future field control of the *B. oleae* female, the scourge of olive oil production in the Mediterranean area.

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TERPENE VARIATIONS IN MARITIME PINE CONSTITUTIVE OLEORESIN RELATED TO HOST TREE SELECTION BY *Dioryctria sylvestrella* RATZ. (*Lepidoptera: Pyralidae*)

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Abstract—Gas chromatographic analysis of the volatile terpenes of the wood oleoresin of maritime pines showed that the relative proportions of terpenes within the same tree did not vary according to sampling height and date. Pruning and tree vigor did not affect terpene profiles, but oleoresin composition varied widely from tree to tree. The trees attacked by *Dioryctria sylvestrella* contained a significantly higher percentage of limonene, longipinene, and copaene, and a discriminant analysis, using the relative percentage of six terpenes, significantly separated infested and uninfested trees. The olfactory sensitivity of adult moths to a series of 20 volatile chemicals, including maritime pine oleoresin components, was evaluated with the electroantennogram method. Both sexes were sensitive to monoterpenes, oxygenated terpenoid compounds, and oleoresin samples. The use of terpenes as host attractants and biochemical markers for resistance to *D. sylvestrella* is discussed.

Key Words—Pine tree, *Pinus pinaster*, Lepidoptera, *Dioryctria sylvestrella*, oleoresin, terpenes, host selection, resistance marker.

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INTRODUCTION

The European stem borer, *Dioryctria sylvestrella* Ratz., is one of the major pests of maritime pines in Europe. This oligophagous insect is restricted to the genus *Pinus* in Eurasia (Speight and Speechly, 1982; Menassieu et al., 1989) with occasional damage to *Picea* spp. (Menassieu, personal communication). Larvae tunnel through the bark into the phloem where they feed from July to October and March to May. If larvae succeed in girdling the stem, the tree may suffer from interruption of sap flow, resulting in a decrease of the annual increment or in the malformation of the trunk and increasing the risk of windbreak (Baronio and Butturini, 1988). Foraging larvae cause resin pockets, which render the timber worthless, particularly for peeling and sawing. For the last 10 years, major infestations of *D. sylvestrella* have developed in southwestern France (Menassieu et al., 1989; Jactel et al., 1994), a region that contains the largest unit of managed forest in Europe (1.2 million ha). The high cost of the intensive management of this maritime pine forest makes it necessary to prevent stems borers from reducing the volume and the quality of the forest products grown. The impossibility of chemical control because the insect is hidden under the bark more than 11 months each year and the uncertainty of biological controls led us to promote changes in silviculture practices and to develop pest-resistant trees. Both of these two strategies are based on the same principle: the reduction of tree attractiveness.

Early work (Jactel et al., 1994, 1995) demonstrated that the infestation rate of *D. sylvestrella* increases with the severity of pruning and with the vigor of maritime pine trees, i.e., with two factors closely linked to intensive pine forest management. Planting at low densities and fertilizing were developed to favor fast growth. Tree pruning, the removal of live branches, is used to reduce the incidence of knots and to increase the proportion of clear lumber in the logs. It was hypothesized that primary attraction, mediated by components of the oleoresin exuding from the pruning wounds or bark cracks, might attract insect to host trees. Our studies suggest that the olfactory system of *D. sylvestrella* females is tuned to volatiles from the constitutive oleoresin, among which terpenes or their derivatives may play a major role in the attraction. Terpenes affect the behavior of a variety of insects (reviewed by Speight and Wainhouse, 1989), including several *Dioryctria* species (Hanula et al., 1985). Moreover, infestation rates of forest insects (Smith, 1966; Annila and Hiltunen, 1977; Rappaport et al., 1995) or variations in tree resistance (Hanover and Furniss, 1966; Wright et al., 1975; Sturgeon, 1979; Charles et al., 1980; Bridgen and Hanover, 1980) have been related to terpene ratios in oleoresin or volatile extracts.

Due to low natural densities and lack of mass rearing, few adult moths were available for laboratory experiment. This made the use of behavioral tests too difficult for studying the attractiveness of terpenes. It seemed more conve-

nient to base our investigations on epidemiological data by comparing the terpene composition of oleoresin between attacked and unattacked trees. Before attacks could be related to individual attractiveness, five precautions had to be taken. First, oleoresin was sampled before *D. sylvestrella* attack. Second, oleoresin was collected from the trunk of maritime pine trees, because the resin involved in tree attractiveness seemed to be that exuding from pruning wounds (Jactel et al., 1994, 1995). This resin is synthesized in the wood (secondary) tissues and stored in resin ducts (Pauly, 1962; Bernard-Dagan, 1968) as opposed to the resin located in cortical (primary) tissues (Baradat and Marpeau-Bezard, 1988). Third, the within-tree variability of the terpene composition had to be determined as a function of sampling height and date. Fourth, since more vigorous trees and pruning are known to increase the risk of attack, it had to be verified whether these physiological changes modify the terpene composition (i.e., the nature of the attractive signal) or only the rate of release (i.e., the emission pattern of the attractive signal). Terpenes whose proportion significantly differ between attacked and unattacked trees might serve as biochemical markers for resistance to *D. sylvestrella*. In a fifth step, we had to determine whether these terpenes act as host attractants themselves. We thus compared, by electroantennography (EAG) recordings, the detection abilities of the European stem-borer moth towards several terpenes and volatiles that commonly occur in pine trees.

METHODS AND MATERIALS

Oleoresin Sampling. Oleoresin samples were taken from 55 twelve-year-old pine trees (*Pinus pinaster* Ait.), unattacked by *D. sylvestrella*: 25 trees were randomly chosen from one pure stand and 30 from a second pure stand grown on damp heaths in the Landes de Gascogne (southwestern France) where the sensitivity of pruned maritime pines has been studied in relation to tree vigor and date of pruning (Jactel et al., 1995). Forty-three trees were pruned at different dates, and 12 remained unpruned until June 1993 at the start of the insect attack period (Table 1). The latter 12 trees (control trees) were pruned in June 1994 in order to study the short-term effects of pruning on the terpene profile. Pruning consisted of the removal of all branches except the upper (younger) four whorls, about 5 m above ground level, which is the usual practice for maritime pine in France. Due to the spacing of trees (almost 2 m) and the number of large branches, pruning is needed to reduce the incidence of knots in saw timber. In October 1993, the diameter at breast height (at 1.3 m above the ground) and the presence of *D. sylvestrella* attacks were recorded on all 55 trees.

A sampling method was developed to collect sufficient wood resin. Holes

TABLE 1. SAMPLE SIZE OF FIVE TREATMENTS APPLIED TO 55 TREES SAMPLED FOR WOOD RESIN COMPOSITION AND *Dioryctria sylvestrella* ATTACK

Tree treatment	Number of trees		
	Stand 1	Stand 2	Total
Pruned in August 1992	4	3	7
Pruned in November 1992	4	4	8
Pruned in February 1993	6	8	14
Pruned in May 1993	6	8	14
Unpruned (control)	5	7	12
Total	25	30	55

were bored with a drill, along an oblique line, through the sapwood, in the vicinity of a branch insertion point. One end of a plastic tube was introduced into the hole and the other side was cellotaped to a glass vial. Oleoresin flowing out of the holes was collected for 24 hr. The vial samples were then placed in a nitrogen atmosphere in Dry Ice, and stored in the laboratory at −30°C until analysis (Delorme and Lieutier, 1990). In each pine stem, constitutive oleoresin was sampled at two levels. One hole was bored near the branch insertion of the 3-year-old internode, where the highest number of *D. sylvestrella* attacks are located (Jactel et al., 1994), and the second hole was bored in the 5-year-old internode, the upper whorl from which live branches were removed in pruned trees and where *D. sylvestrella* attacks also occur. In control trees, oleoresin was sampled seven times at the same two levels: in August 1992, November 1992, February 1993, May 1993, June 1993, and in June 1994 before and after pruning (14 samples per control tree). In pruned trees, oleoresin was sampled once near the pruning wounds the day after pruning and at the two heights defined above in June 1993 (three samples per pruned tree).

Gas Chromatographic Analyses. Only mono- and sesquiterpenes were studied. Approximately 1 ml of resin was solubilized in 1.5 ml of pentane. Pentane solutions of the terpenes were purified by column chromatography on SDS silica 70–230 mesh (8 ml) in a glass column (20 cm long × 1 cm ID). Terpenes were eluted by a pentane–ether solution (30 ml, 98:2), and sodium sulfate (0.5 ml) was added to eliminate water from the resin extract. The purified extract was concentrated by evaporation under moderate vacuum and analyzed by FID-GC (Delsi Intersmat IGC-120, temperature program. 60°C to 280°C at 6°C/min, splitless (injector 200°C, detector 290°C) on a 25-m × 0.25-mm-ID Chrompack CPSIL 5 column (N_2, 0.4 ml/min, 1 μl sample). The components had been previously identified by infrared and mass Spectrometry and by comparing their

retention times with those of authentic samples (Pauly et al., 1973). The relative percentages of mono- and sesquiterpenes were calculated from the summation of all recorded terpene peaks.

Electroantennography. D. sylvestrella chrysalids were collected from attacked pine trees near the INRA Forestry Centre (Gazinet, France) in May–June 1994. Adults emerged in the laboratory and were maintained in containers at 20°C under natural light. Three- to 6-day-old moths of both sexes were used for electrophysiological recordings. Electroantennograms (EAG) were recorded from 6 males and 11 females with a classical electrophysiological set-up (Gabel et al., 1992). Head antenna preparations were stimulated with a series of volatile compounds delivered for 2 sec (2 ml/sec) in a continuous airflow (4 ml/sec; humidified, compressed air). A variable time interval (30 sec to 2 min.) enabled the antenna to recover from previous stimulation. All chemicals were diluted in paraffin oil (10^{-2} v/v) and deposited on filter paper (5 μl on 2 cm^2) inside a disposable Pasteur pipet. A standard stimulus, hexanol, was delivered every four stimulations. Hexanol was chosen as a standard because it is a general green leaf volatile (Visser, 1986). Chemical compounds (GC purity) were either purchased from commercial sources or prepared from natural maritime pine oleoresin. The series of 20 compounds tested included mono- and sesquiterpenes, terpenic acetates, terpenic alcohols, total oleoresins, and paraffin oil and blank (empty pipet) as controls (listed in Figure 3 below).

Electrical signals were continuously recorded and analyzed on a PC-compatible microcomputer using a custom-designed program (Marion-Poll and Tobin, 1991; Marion-Poll and Thiéry, 1995). EAG responses were expressed relative to the response of the standard stimulus (hexanol).

Statistical Analyses. All statistical analyses were carried out with SAS Software (SAS Institute Inc., 1987). Analyses of variance (*t* test or GLM) of percentage variables were computed with the arcsin $\sqrt{x}$ transformation (Dagnelie, 1973). Mean differences were tested using Scheffé's multiple range test.

RESULTS

Within-Tree and Intertree Variations in Terpene Composition. Thirteen terpenes exhibited relative proportions higher than 0.05%, and were used throughout the study. In order of increasing retention time, there were six monoterpenes: α-pinene, camphene, β-pinene, myrcene, limonene, terpinolene; and seven sesquiterpenes: longipinene, copaene, cubebene, longifolene, β-caryophyllene, α-humulene and germacrene. The α- and β-pinenes averaged 90% of the total amount of terpenes and the sesquiterpenes almost 5%. The relative proportions of terpenes determined by gas chromatography were accurate since the standard error varied from 0.4% for α-pinene, the major com-

ponent (60%), to 0.003% for copaene and germacrene, the minor components (0.1%).

The variances of the relative proportions of the 13 terpenes in the 12 unpruned trees were analyzed according to sampling date (five dates in 1993, one date in 1994 before pruning, $df = 5$), sampling height (3- and 5-year-old internodes, $df = 1$), and the interaction between these factors ($N = 144$). None of the factors nor their interaction induced any significant differences ($P > 0.05$). The terpene composition of the oleoresin did not vary with sampling height in the part of the stem where *D. sylvestrella* attacks are located. Only minor changes were recorded in the relative proportion of terpenes during the same year and between two successive years (Figure 1). In June 1994, the control trees were sampled before and 48 hr after pruning, at both heights. There were no significant differences for any of the 13 terpenes between the relative proportion before and shortly after pruning (t test, $N = 12$, $P > 0.05$). Likewise, the 43 pruned trees were sampled in the 5-year-old internode 48 hr after pruning and in June 1993. There were no significant differences for any of the 13 terpenes between the relative proportion just after pruning and 1–10 months after pruning (t test, $N = 7$ to $N = 14$, $P > 0.05$).

The percentage of each terpene showed a wide intertree variability. The 12 unpruned trees and the 43 pruned trees differed significantly from each other in all components of their terpenes composition ($N = 12$ and $N = 3$, respectively; $P < 0.001$). For both pruned and unpruned trees, the coefficient of variation of the terpenes proportions was 1.2- to 7.5-fold more important between trees than within trees (Table 2). Simple correlation coefficients of each terpene proportion with the diameter at breast height were computed for all 55 trees. Terpene proportions were never significantly correlated with tree diameter ($N = 55$, $P > 0.05$).

Terpene Composition and Susceptibility to D. sylvestrella *Attack.* Since pruning did not induce any change in terpene composition, the 12 unpruned trees and the 43 pruned trees were pooled for this analysis. Thirty-three trees were attacked and 22 were uninfected. Wood resins did not differ qualitatively between attacked and unattacked trees, since both types of tree produced the same typical chromatogram of the same 13 terpenes. However, some differences in relative percentage of terpenes were significant (Figure 2). Trees that had been attacked exhibited significantly more limonene ($P < 0.04$), longipinene ($P < 0.03$), and copaene ($P < 0.03$) and less camphene ($P < 0.004$) than unattacked trees. The ratio of limonene plus longipinene plus copaene to camphene differed even more significantly between attacked and unattacked trees ($P < 0.003$). Discriminant analyses were performed to determine whether the whole terpene composition could separate the two classes of trees. The best discriminant analysis was obtained with the combination of six variables: the proportion of β-pinene, camphene, myrcene, limonene, terpinolene, and ger-

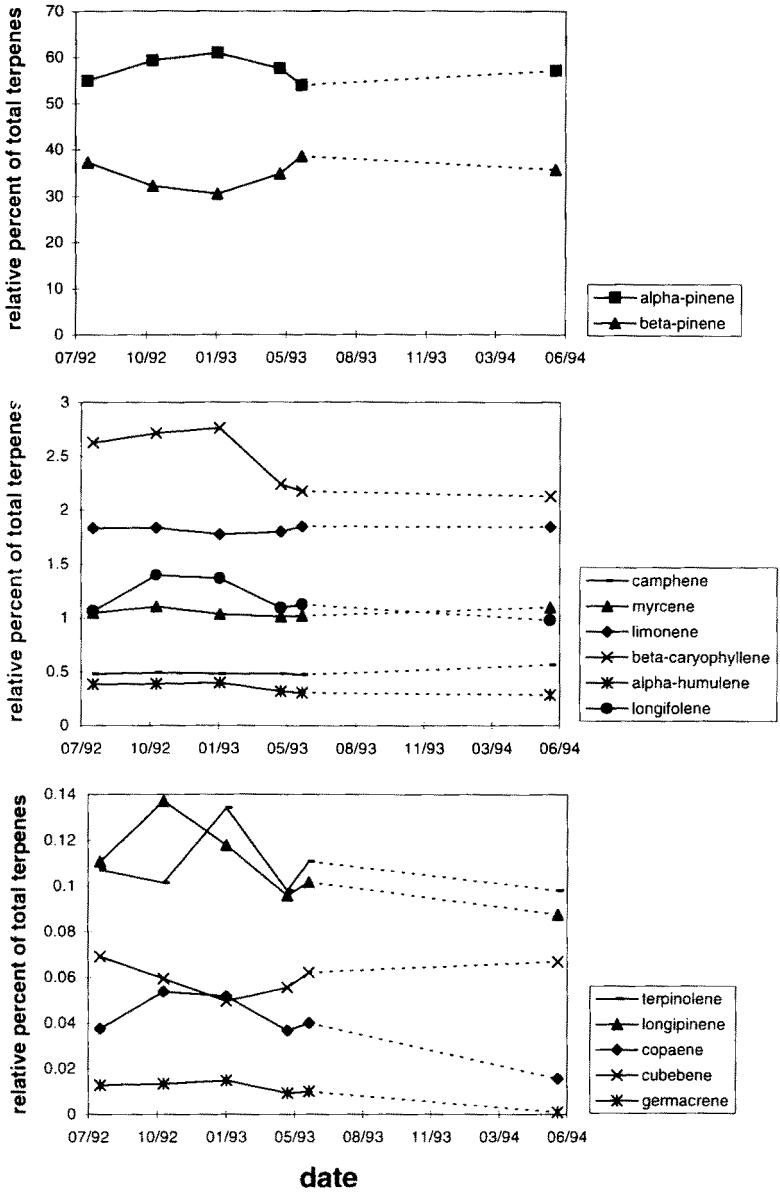

FIG. 1. Changes in the relative percentage of terpenes in the wood resin of maritime pines over the course of two years (12 trees).

TABLE 2. WITHIN AND INTERTREE COEFFICIENT OF VARIATION (CV) OF RELATIVE PROPORTION OF 13 TERPENES PRESENT IN MARITIME PINE WOOD RESIN

	Terpene[a]												
	API	CAM	BPI	MYR	LIM	TPL	LGP	COP	CUB	LGF	BCA	AHU	GER
Control trees[b]													
Within-Tree	6.6	6.5	13.2	7.3	5.1	39.2	26.4	37.4	45.7	26.4	26.0	27.4	111.2
Intertree	18.9	7.7	31.4	8.7	8.5	60.2	77.1	112.1	47.4	96.9	41.8	36.0	73.3
Pruned trees[c]													
Within-tree	4.7	9.0	10.5	8.2	4.3	29.8	39.6	57.4	28.9	49.2	24.2	25.8	55.5
Intertree	19.0	20.2	36.6	14.8	10.4	89.8	104.1	143.7	58.1	133.8	53.8	50.3	418.6

[a] API, α-pinene; CAM, camphene; BPI, β-pinene; MYR, myrcene; LIM, limonene; TPL, terpinolene; LGP, longipinene; COP, copaene; CUB, cubebene; LGF, longifolene; BCA, β-caryophyllene; AHU, α-humulene; GER, germacrene.

[b] Control trees: within tree CV is calculated as the mean (N = 12 trees) of the CV of the proportion of terpenes in each tree (N = 12, two heights, six dates). Intertree CV is calculated as the CV (N = 12 trees) of the mean proportion of terpenes in each tree (N = 12, two heights, six dates).

[c] Pruned trees: Within tree CV is calculated as the mean (N = 43 trees) of the CV of the proportion of terpenes in each tree (N = 3, two heights, two dates). Intertree CV is calculated as the CV (N = 43 trees) of the mean proportion of terpenes in each tree (N = 3, two heights, two dates).

macrene. Wilk's lambda statistic showed a highly significant difference between the means of attacked and unattacked trees ($F = 3.19$, $P = 0.007$). The resubstitution using linear discriminant function produced an error count estimate of 28% for the attack (18.2% for misclassified attacked trees and 37.9% for misclassified unattacked trees).

Electrophysiological Response of D. sylvestrella *to Terpenoid Compounds.* All compounds elicited EAG responses within 20–140% of the hexanol standard EAG responses of both sexes were compared with a t test. Eight compounds elicited significantly higher responses in females: α-pinene ($P < 0.02$), camphene ($P < 0.003$), β-pinene ($P < 0.02$), myrcene ($P < 0.04$), δ3-carene ($P < 0.02$), limonene ($P < 0.04$), longifolene ($P < 0.03$), β-caryophyllene ($P < 0.002$). Although differences in mean EAG response to the 21 compounds were tested separately, their patterns were the same in males and females (Figure 3). The most stimulating substances were pine tree resins, 4-terpineol, linalool, and neryl acetate. Medium stimuli were given by the monoterpenes and geraniol. Sesquiterpenes, terpenyl acetate, and nerolidol did not induce any response that was significantly different from those elicited by the blank and the paraffin oil.

DISCUSSION

Terpene Variations in Constitutive Oleoresin. The terpene composition of the sampled resin, with pinenes representing almost 90% of the total amount,

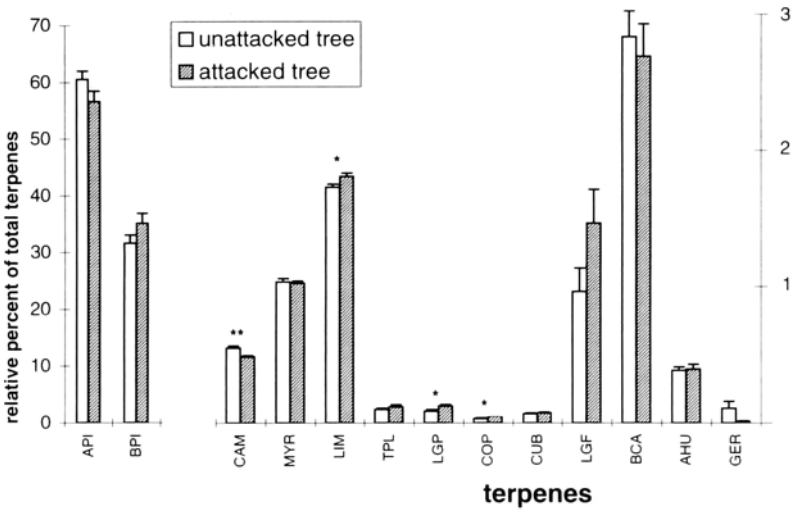

FIG. 2. Comparison of mean (± standard deviation) terpene profiles of wood resin in attacked and unattacked maritime pine trees. One or two stars above a pair of bars indicate a significant or highly significant difference between the mean relative percentage of terpene in attacked and unattacked tree. API: α-pinene, BPI: β-pinene, CAM: camphene, MYR: myrcene, LIM: limonene, TPL: terpinolene, LGP: longipinene, COP: copaene, CUB: cubebene, LGF: longifolene, BCA: β-caryophyllene, AHU: α-humulene, and GER: germacrene.

was very similar to the composition of the secondary resin already described in maritime pine (Bernard-Dagan, 1968; Baradat and Marpeau-Bezard, 1988). Collecting resin from holes bored through the sapwood may thus provide a convenient method for assessing the chemical composition of the oleoresin flowing out of the pruning wounds, which are particularly damaging to the secondary tissues. Our data suggest that pruning has neither short-term nor long-term effects on the terpene composition of the resin. However, it has been reported that mechanical injuries increase the resin content of pine tissues, due to the induced defense reaction (Berryman, 1972; Christiansen and Hornvendt, 1983; Lieutier et al., 1989). In pruning wounds, the resin exuding from several resin ducts might mix with the resin of the induced reaction, but it has been demonstrated that the terpene composition of the neosynthesised resin is very similar to that of wood resin (Cheniclet, 1987; Baradat and Marpeau-Bezard, 1988). The mixture of resins exuding from pruning wounds would thus be of the secondary type. Therefore, if terpenes in exuding resins were involved in the host attraction of *D. sylvestrella* they can be profitably studied in the secondary resin known as wood resin.

The terpene composition of wood resin did not vary significantly within

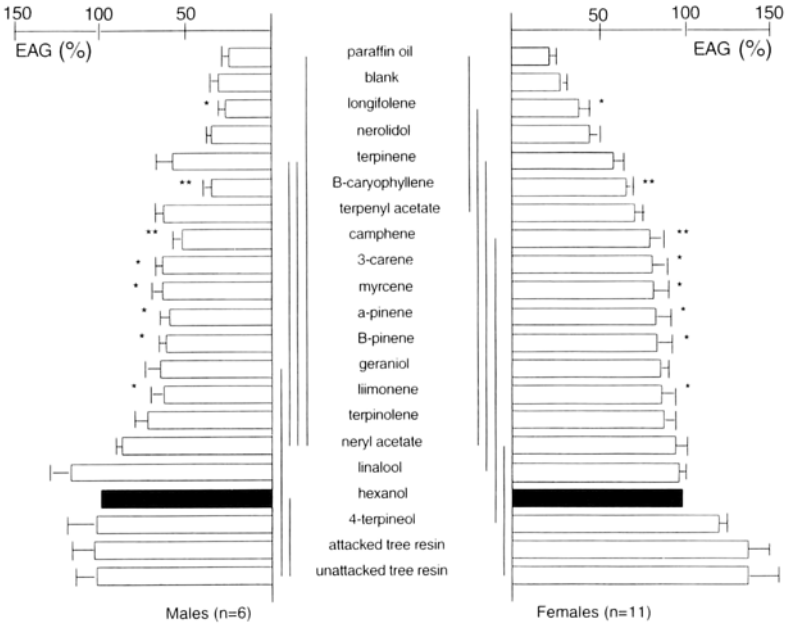

FIG. 3. Male and female *Dioryctria sylvestrella* EAG responses relative to the standard (hexanol). Bars represent the EAG relative amplitude (mean ± standard deviation). Stars indicate a highly significant difference between male and female EAGs. Two substances joined by the same vertical line (Scheffé's multiple range test) do not elicit a significantly different EAG response (lines at the left for males, at the right for females).

the same trunk, which supports previous findings that there is no variation in the wood resin composition according to the sampling height (Bernard-Dagan, 1968; Bernard-Dagan et al., 1971; Baradat and Marpeau-Bezard, 1988). Likewise, in *Pinus pinaster* (Bernard-Dagan et al., 1971), *Pinus monticola* Dougl. (Hanover and Furniss, 1966), or *Picea glauca* (Moench) Voss. (von Rudloff, 1972) the relative proportions of monoterpenes in individual trees change only slightly between seasons in shoot or stem tissues. In wood tissues, the terpene composition of the resin stabilized towards the fifth year of growth (Baradat et al., 1972; Schmitt et al., 1988). Consequently, collecting only one wood resin sample anywhere in the trunk and at anytime provides a convenient way of assessing the terpene profile of a single tree.

The 55 pine trees sampled in this study differed significantly with respect to the relative proportion of each of the 13 terpenes detected in the constitutive resin. This pattern of wide intertree variability in terpene composition has been noticed in the resin of many conifers, including *P. pinaster* (Bernard-Dagan,

1968), *Pinus muriatica* D. (Mirov et al., 1966), *P. contorta* (Pauly and von Rudloff, 1971), *Pinus ponderosa* (Zavarin et al., 1971), and *Picea abies* L. Karst (Schönwitz et al., 1987). In agreement with Douglas fir studies by Hanover and Furniss (1966), we found no significant correlation between the relative percentage of terpenes and tree diameter. As a rule, this intertree variability probably mainly results from genetic control (Hanover, 1966; Mirov et al., 1966; Squillace et al., 1980; Baradat and Marpeau-Bezard, 1988). The terpenes of wood resin are stable in time and space within the same stem, variable between trees, and under genetic control. Thus they possess all the attributes that would make them biochemical markers for selecting genetic resistance to *D. sylvestrella*.

Terpenes as Biochemical Markers for Resistance to D. sylvestrella. For terpenes to be used as biochemical markers for tree resistance, they have to be consistently related to the intensity of damage. The percentage of cones infected by *Pissodes validirostris* is significantly correlated with the α-pinene extracted from needles (Annila and Hiltunen, 1977), and the attack rates of *Megastigmus spermotrophus* Wachtl increase significantly with limonene and terpinolene levels in *Pseudotsuga menziesii* Mirb. cones (Rappaport et al., 1995). Wright et al. (1975) related the resistance of southern Scots pine varieties to *Dioryctria zimmermanni* (Grote) to low levels of δ 3-carene and terpinolene. In this study, Maritime pine sensitivity to *D. sylvestrella* was significantly correlated with high level of limonene, longipinene, and copaene and low levels of camphene in wood resin. Ratios of four terpenes and discriminant analysis, involving six different terpenes, further improved the significance of the differences between attacked and unattacked pine trees. This result seems to be consistent with the way polyphagous insects perceive host plants, which is believed to be based on mixtures or ratios of common volatiles rather than specific molecules (Visser, 1986; Marion-Poll and Thiéry, 1995).

The behavioral role of the wood resin terpenes has not yet been established in *D. sylvestrella*, but the physiological sensitivity of the species was assessed by EAG. As a rule, the EAGs from females were greater in amplitude (relative to the standard) than those of males. Since sexual dimorphism in the number of head antenna receptors may exist, further anatomical observations are needed to interpret these differences. Sesquiterpenes did not induce significant electrophysiological response, but monoterpenes were stimulating and oxygenated monoterpenes (alcohols and acetates) were even more effective. It is noteworthy that the amplitude of adult moth responses increased with the relative volatility of the tested compounds. Marion-Poll and Thiéry (1995) found the same order in *M. spermotrophus*, but this might be due to a technical artifact. Because head antenna preparations were stimulated by volatile compounds via a series of constant air volumes, the number of molecules that reach the antennal receptors was directly proportional to compound volatility. Hence, the more volatile compounds may be more stimulative.

The maximum response was elicited by the odors of complete wood resins. These extracts might contain a great number of unknown and untested stimulating compounds. The amplitude of the overall response may have been higher merely because of the additive effects of the various components. Moreover, no particular signal compound seemed to be distinguishable from the others by an especially high amplitude of response. These observations suggest that the olfactory system of *D. sylvestrella* is tuned to perceiving attractant mixtures or ratios of commonly occurring pine volatiles rather than to detecting specific compounds. These perception features could then explain why ratios of terpenes and discriminant analysis improved the significance of differences between infested and uninfested trees.

In conclusion, this study pointed out that monoterpenes of the wood resin may provide suitable markers in indirect selection programs for *D. sylvestrella* resistance in maritime pine, provided that heritability of the terpene profile can be demonstrated. In order to improve our understanding of host tree selection and to evaluate the potential use of behavior-modifying chemicals in pest management, headspace volatiles analyses and behavioral experiments should be developed to determine the most effective host attractants. Our data suggest that particular attention should be paid to monoterpenes and oxygenated terpenoid compounds.

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CHEMICAL DEFENSE OF GIANT SPRINGTAIL *Tetrodontophora bielanensis* (WAGA) (INSECTA: COLLEMBOLA)

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Abstract—The giant springtail, *Tetrodontophora bielanensis* (Waga), is characterized by integumental openings (pseudocells) from which small droplets of a sticky defensive fluid are secreted after molestation. The secretion originates initially from secretory cells below the pseudocellae; subsequent irritations result in release of hemolymph, which was identified by both chemical and microscopical methods as well as by scanning electron microscopy. Bioassays with topically treated ground beetles *Nebria brevicollis* showed that the pseudocellular fluid evokes a total disorientation and cleansing behavior of the beetle. The main constituents were identified as the following pyridopyrazines: 2,3-dimethoxypyrido[2,3-b]pyrazine (**1**), 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine (**2**), and 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (**3**). These alkaloids are mainly present in the pseudocellar fluids of female and male springtails but are absent in their food or feces. Minor amounts are found in the hemolymph of adults, while larvae contain traces of **2** only. All compounds were synthesized and tested for activity. In natural concentrations, the synthetic alkaloids elicited the same effects from the ground beetles as the pseudocellar fluid.

Key Words—Chemical defense, alkaloids, pyridopyrazines; Collembola, *Tetrodontophora bielanensis*, pseudocells, cleansing behavior, disorientation, 2,3-dimethoxypyrido[2,3-b]pyrazine, 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine, 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one.

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INTRODUCTION

Apterygote insects represent an ecologically important group of mainly soil-, bark-, litter-, and moss-inhabiting arthropods that occur in a wide range of moist environments. With maximal densities of 53,000 individuals m^2, springtails (Collembola) in particular are often exceedingly abundant and are among the most important consumers in many soil ecosystems. Springtails and other apterygotes also represent important and readily available prey for many predatory mites, spiders, beetles, bugs, or hymenopteran species. According to the primitive character of this large group of mainly entognathous hexapods, apterygote insects were recently considered to be devoid of chemical defenses (Berenbaum and Seigler 1992). However, a careful literature survey amply reveals that chemical defense is widespread in both ento- and ectognathous hexapods. The weakly sclerotized Protura contain voluminous abdominal defensive glands and, like staphylinid beetles, can bend their abdominal tips toward the aggressor to emit an acidic sticky gland material (Berlese, 1909; Francois and Dallai, 1986). When molested springtails escape from potential predators by suddenly releasing their furca, which is held under tension beneath the abdomen, thus propelling themselves through the air. Many species of the eyeless soil-inhabiting Onychiuridae have lost their jumping ability for escape and instead may release sticky droplets of unknown chemical composition from small integumental openings called pseudocells (Hale and Smith, 1966; Koncek, 1924). These pseudocellar fluids may mechanically repel predators by contaminating their body and appendages. In *Onychiurus* species these fluids exhibit paralyzing activities on predatory mites (Karg, 1961) and effectively repel other springtails and millipedes (Simon, 1961). Aromatic alkaloids, 2,3-dimethoxypyrido[2,3-b]pyrazine (**1**) and 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine (**2**), were identified in the extracts of whole *Tetrodontophora bielanensis* (Budesinsky et al., 1986). Since it has not yet been clarified whether the pseudocellar fluid of onychiuran Collembola represents a true secretion or simply hemolymph, we want to report here on the origin and depletion of these droplets in the giant (length 5–9 mm) collembolan species *Tetrodontophora bielanensis* (Waga). Moreover, unambiguous structural assignments of biologically active constituents of this fluid will be presented as well as bioassays using pseudocellar fluids and synthetic compounds in order to demonstrate the defensive activity of the constituents.

METHODS AND MATERIALS

Individuals (630) of the giant collembolan species *Tetrodontophora bielanensis* (Waga) were collected and sieved either from a beech-forest litter of the Czech Elbsandsteingebirge near Hrensko (altitude 250 m) or from litter of a pine–beech forest near Cierny Balog (Tatra, Slovakia; altitude 900 m). In the

laboratory most of the specimens were frozen or kept alive in small plastic boxes supplied with moistened plaster bottoms and soil (constant temperature control 12°C; light–dark 8:16). The springtails were fed with dried dog food and granulated pollen.

Whole specimens or dissected body parts of *T. bielanensis* were transfered to Gisin's fixation mixture (Gisin, 1960), dehydrated in a graded series of acetone, critical-point dried with carbon dioxide, and sputter coated with gold. Scanning electron microscopy (SEM) was carried out by using a Cambridge Stereoscan 90. For examination of the cuticular parts, fragments of *T. bielanensis* were previously treated with 5% potassium hydroxide for 24 hr.

Tiny droplets of pseudocellar fluid were secreted after slight molestations of springtails with a forceps, and the droplets were collected with microcapillaries. In order to obtain hemolymph, the collophore of previously frozen springtails was dissected and hemolymph samples were taken with a microcapillary. Collected fluids were used for microscopical examination (photomicroscope Olympus BH-2), Wright-staining (Clark, 1973), electrophoresis, GC-MS examination, and bioassays. One microliter of pseudocellar fluid or hemolymph of *T. bielanensis* was employed for each electrophoretic separation. For native disk PAGE the sample was mixed with 20 μl buffer (10 mM Tris HCl, 1 mM EDTA, 0.01% bromophenolblue), centrifuged at 13,000*g* for 30 min, and the supernatant diluted with buffer to give a sample–buffer ratio of 1:80 prior to electrophoresis. Electrophoresis was carried out at 10°C using the PhastSystem and PhastGel Gradient 8-25 (both Pharmacia). The gel was developed by silver staining (method 3 according to PhastGel Silver Kit instruction manual). For SDS disk PAGE samples were mixed with SDS-buffer (2.5% SDS, 5% mercaptoethanol), heated for 5 min at 80°C, centrifuged, diluted with SDS buffer, separated, and stained as above.

The ^{1}H and ^{13}C NMR spectra were obtained with Bruker WM 400 and AC 250P instruments with TMS as an internal standard. Mass spectra (70 eV) were obtained with a VG 70/250 S mass spectrometer coupled to a Hewlett-Packard HP 5890 A gas chromatograph. Gas chromatography was carried out with a Carlo-Erba Fractovap 2101 instrument equipped with a flame ionization detector and on-column injection. Separations were performed using a 30-m DB-5 (0.32 mm internal diameter (ID), thickness of decoding film (d_f) = 0.25 μm) fused silica column with hydrogen as the carrier gas. Melting points (uncorrected) were measured with an Ernst Leitz instrument. Column and thin-layer chromatography were performed on silica gel (70-230 mesh, Merck).

Syntheses of Pyridopyrazines 1–3

*1H,4H-pyrido[2,3-b]pyrazine-2,3-dione (**B**).* This alkaloid was prepared from 2,3-diaminopyridine (**A**) according to the method of Winterfield and Wildersohn (1970).

2,3-Dichloro-pyrido[2,3-b]pyrazine (E). A mixture of 320 mg (2 mmol) **B**, 1 ml $POCl_3$, and 200 mg triethyl amine was heated under reflux for 2.5 hr. The mixture was treated with ice water, neutralized with aq. NaOH solution and extracted with chloroform. After drying over $MgSO_4$ and removal of the solvent, the product was purified by column chromatography (silica) to yield 320 mg (80%) of the pure product. mp: 149°C [Lit. 144–145°C (Winterfeld and Wildersohn, 1970)]. ^{1}H NMR (250 MHz, $CDCl_3$): δ = 7.79 (dd, 1H, H-7, $J_{6,7}$ = 4.2 Hz, $J_{7,8}$ = 8.4 Hz), 8.42 (dd, 1H, H-8, $J_{6,8}$ = 1.8 Hz), 9.29 (dd, 1H, H-6). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 126.29 (C-7), 130.18 (C-8a), 137.20 (C-8), 146.50 (C-4a), 148.75 (C-3), 154.13 (C-2), 154.92 (C-6).

2,3-Dimethoxy-pyrido[2,3-b]pyrazine (1): A mixture of 800 mg **E** and 5 ml 2 M sodium methoxide solution was heated under reflux for 1 hr. After cooling, the mixture was treated with water and extracted three times with chloroform. The combined organic phases were dried over $MgSO_4$ and the solvent removed. Column chromatography (silica, ethyl acetate–methanol 1 : 1) yielded the pure alkaloid **1** in 53% yield (400 mg). mp: 125°C. ^{1}H NMR (250 MHz, $CDCl_3$): δ = 4.18 (s, 3H, CH_3), 4.26 (s, 3H, CH_3), 7.48 (dd, 1H, H-7, $J_{6,7}$ = 4.4 Hz, $J_{7,8}$ = 8.0 Hz), 8.15 (dd, 1H, H-8, $J_{6,8}$ = 1.8 Hz), 8.78 (dd, 1H, H-6). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 54.76 (q, CH_3, J = 147.8 Hz), 55.10 (q, CH_3, J = 147.8 Hz), 122.31 (C-7, J = 165.0 Hz, J = 9.0 Hz), 132.06 (C-8a, J = 8.6 Hz), 135.07 (C-8, J = 164.0 Hz, J = 6.4 Hz), 147.89 (C-4a, J = 13.0 Hz, J = 5.2 Hz), 149.06 (C-6, J = 179.3 Hz, J = 9.0 Hz, J = 3.3 Hz), 150.39 (C-3, J = 3.8 Hz), 152.39 (C-2, J = 3.8 Hz). The mass spectrum and formula for compound **1** are presented in Figure 5 below.

3-Isopropyl-1H-pyrido[2,3-b]pyrazine-2-one (F). A mixture of 1 g (9.3 mmol) **A** and 2 g (10.4 mmol) methyl 3-methyl-2-oxobutyrate (3 : 1 mixture with diethyl oxalate) (Weinstock et al., 1981) were heated under nitrogen for 1 hr to 100°C. After cooling, the residue was washed successively with pentane, chloroform and ethanol. A mixture of the regioisomeric ketones 3-isopropyl-1*H*-pyrido[2,3-b]pyrazine-2-one (**F**) and 2-isopropyl-4*H*-pyrido[2,3-b]pyrazine-3-one (**G**) was present in the chloroform and ethanol phases; the insoluble residue represents the dione **B.** After combining the chloroform and ethanol phases and removing the solvents, the ketones were separated by column chromatography (silica, 0.5% NH_4OH, ethyl acetate). 3-Isopropyl-1*H*-pyrido[2,3-b]pyrazine-2-one (**F**) R_f = 0.09 (ether), mp: 254–256°C, yield: 540 mg (31%). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.37 (d, 6H CH_3, J = 6,8 Hz), 3.70 (sept, 1H, CH), 7.46 (dd, 1H, H-7, $J_{6,7}$ = 4.6 Hz, $J_{7,8}$ = 8.4 Hz), 7.75 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 8.67 (dd, 1H, H-6), 12.5 (bs, 1H, NH). MS (70 eV): m/z(%) = 39 (18), 64 (14), 65 (17), 78 (6), 91 (12), 92 (12), 93 (12), 119 (13), 120 (14), 133 (10), 146 (100), 160 (27), 161 (50), 174 (78), 189 (62, M^+).

2-Isopropyl-4*H*-pyrido[2,3-b]pyrazine-3-one (**G**) R_f = 0.67 (ether), mp: 193–194°C, yield: 720 mg (41%). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.34 (d, 6H, CH_3, J = 6,8 Hz), 3.64 (sept, 1H, CH), 7.33 (dd, 1H, H-7, $J_{6,7}$ = 4.8 Hz, $J_{7,8}$ = 8.0 Hz), 8.17 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 8.59 (dd, 1H, H-6), 12.5 (bs, 1H, NH). MS (70 eV): m/z(%) = 39 (18), 64 (10), 66 (12), 78 (8), 93 (14), 120 (22), 133 (8), 146 (100), 161 (87), 174 (58), 189 (75, M^+).

3-Isopropyl-2-methoxy-pyrido[2,3-b]pyrazine (2). As described above in the synthesis of **1,** the ketone **F** was converted into 2-chloro-3-isopropyl-pyrido[2,3-b]pyrazine [**H,** 85% yield; ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.49 (d, 6H, CH_3, J = 6,6 Hz), 3.77 (sept, 1H, CH), 7.89 (dd, 1H, H-7, $J_{6,7}$ = 4.4 Hz, $J_{7,8}$ = 8.4 Hz), 8.56 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 9.26 (dd, 1H, H-6)], which was subsequently converted to **2.** ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.41 (d, 6H, CH_3, J = 6.8 Hz), 3.55 (sept, 1H, CH), 4.14 (s, 3H, CH_3), 7.54 (dd, 1H, H-7, $J_{6,7}$ = 4.4 Hz, $J_{7,8}$ = 8.2 Hz), 8.16 (dd, 1H, H-8, $J_{6,8}$ = 1.8 Hz), 8.87 (dd, 1H, H-6). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 20.30 (2 CH_3), 30.78 (CH), 54.14 (OCH_3), 124.16 (C-7), 134.41 (C-8a), 135.51 (C-8), 148.57 (C-4a), 149.29 (C-6), 156.13 (C-2), 159.43 (C-3). The mass spectrum (70 eV) and formula for compound **2** are presented in Figure 5 below.

2-Methoxy-4H-pyrido[2,3-b]pyrazine-3-one (3). A mixture of 1 g (9.1 mmol) **A,** 1.85 g (18.3 mmol) triethyl amine, 1.56 g (8.3 mmol) methyl 2,2-dichloro-2-methoxyacetate and 20 ml absolute toluene was heated under nitrogen to 100°C. After 1 hr the cooled mixture was filtrated and the residue subsequently washed with chloroform and water. The brownish residue contained a 9:1 mixture of the two regioisomers 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (**I** = **3**) and 3-methoxy-1*H*-pyrido[2,3-b]pyrazine-2-one (**II**), which were separated by column chromatography (silica, methanol–triethyl amine, 99:1). The products were insoluble in most organic solvents with the exception of DMSO and pyridine. Structural assignments of the products was achieved by the use of ^{1}H, ^{13}C NMR coupling obtained with gated-decoupled ^{13}C NMR-spectra (see below). 2-Methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (**3**) yield: 420 mg, 34%. ^{1}H NMR (400 MHz, [D_5]-pyridine): δ = 4.02 (s, 3H CH_3), 7.19 (dd, 1H, H-7, $J_{6,7}$ = 5.0 Hz, $J_{7,8}$ = 8.0 Hz), 7.97 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 8.50 (dd, 1H, H-6). ^{13}C NMR (100 MHz, [D_5]-pyridine): δ = 54.65 (OCH_3), 119.58 (C-7), 126.79 (C-8a), 134.08 (C-8), 144.32 (C-4a), 146.69 (C-6), 152.81 (C-2), 156.64 (C-3). The mass spectrum of compound **3** is shown in Figure 5 below. 3-Methoxy-1*H*-pyrido[2,3-b]pyrazine-2-one (**II**) yield: 50 mg, 4%. ^{1}H NMR (400 MHz, [D_5]-pyridine): δ = 4.09 (s, 3H, CH_3), 7.28 (dd, 1H, H-7, $J_{6,7}$ = 4.6 Hz, $J_{7,8}$ = 8.0 Hz), 7.70 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 8.63 (dd, 1H, H-6).

1,4-Dimethyl-1H,4H-pyrido[2,3-b]pyrazine-2,3-dione (C). To a solution of 100 mg (0.63 mmol) **B** in 20 ml absolute DMSO were added 40 mg (1.66 mmol) sodium hydride. A whitish precipitate was formed with hydrogen release.

After the gas formation had ceased, 200 mg (1.4 mmol) methyl iodide were added. The precipitate dissolved, and the solution became reddish. The reaction mixture was then treated with 30 ml brine. After extraction with methylene chloride the combined organic phases were dried with $MgSO_4$. The residue obtained by filtration and removal of the solvent was subjected to column chromatography to yield 60 mg (50%) of a pure product (silica, ethyl acetate/0.5% NH_4OH). ^1H NMR (400 MHz, $CDCl_3$): δ = 3.67 (s, 3H, CH_3), 3.77 (s, 3H, CH_3), 7.28 (dd, 1H, H-7, $J_{6,7}$ = 4.2 Hz, $J_{7,8}$ = 8.0 Hz), 7.59 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 8.30 (dd, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.33 (q, NCH_3, J = 143.0 Hz), 29.21 (q, NCH_3, J = 140.8 Hz), 119.04 (C-7, J = 166.0 Hz, J = 9.0 Hz), 121.56 (C-8, J = 164.1 Hz, J = 6.7 Hz, J = 1.0 Hz), 123.39 (m, C-8a), 138.63 (m, C-4a), 142.16 (C-6, J = 182.2 Hz, J = 7.6 Hz, J = 2.9 Hz), 153.33 (C-2), 154.50 (C-3). MS (70 eV): m/z (%) = 52 (12), 66 (8), 78 (15), 91 (9), 107 (8), 119 (6), 120 (12), 135 (8), 134 (82), 148 (12), 162 (28), 163 (22), 191 (100, M^+).

2-Methoxy-4-methyl-4H-pyrido[2,3-b]pyrazine-3-one (D). The *N*-methylated ketone **D** was obtained from **3** in an identical synthesis as described for **C**. Yield 63%. ^1H NMR (400 MHz, $CDCl_3$): δ = 3.85 (s, 3H, CH_3), 4.15 (s, 3H, CH_3), 7.26 (dd, 1H, H-7, $J_{6,7}$ = 4.2 Hz, $J_{7,8}$ = 8.0 Hz), 7.92 (dd, 1H, H-8, $J_{6,8}$ = 1.6 Hz), 8.43 (dd, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.17 (q, NCH_3, J = 142.8 Hz), 54.90 (q, NCH_3, J = 147.8 Hz), 119.73 (C-7, J = 164.0 Hz, J = 8.6 Hz), 126.76 (C-8a, J = 7.6 Hz), 134.54 (C-8, J = 163.0 Hz, J = 7.0 Hz), 143.17 (m, C-4a), 145.97 (C-6, J = 179.1 Hz, J = 7.6 Hz, J = 2.4 Hz), 152.02 (C-3), 154.77 (C-2). MS (70 eV): m/z(%) = 52 (11), 64 (14), 78 (12), 91 (28), 105 (8), 119 (28), 120 (23), 132 (14), 133 (10), 134 (14), 148 (29), 162 (55), 176 (17), 191 (100, M^+).

2-Quinoxalinole in acetone was used as an internal standard to quantify the total amount of pyridopyrazines in pseudocellar fluids and hemolymph samples (6 samples each) of three specimens of *T. bielanensis* by GC. The presence/absence and semiquantitative determination of the three pyridopyrazines within various compartments, feces, and food of *T. bielanensis* was tested by GC-MS (four samples in each case). Percentage of total peak areas of the pyridopyrazines (total ion chromatogram) reflect their proportion either within the hemolymph or pseudocellar fluids of females, males and larvae.

In order to test the biological significance of *T. bielanensis* pseudocellar fluid on natural predators, interactions of *Nebria brevicollis* ground beetles and the giant springtails were observed and analyzed qualitatively and quantitatively using a videocamera (JVC, GX-N8E). Single individuals of each species were placed within a light-exposed 6 × 6 plastic arena (moistened calcium sulfate bottom) and 20 interactions were recorded qualitatively by using a Panasonic NV-180EG recorder. Quantitation of deterrency was measured by placing 1 μl of pseudocellar fluid (*T. bielanensis*), hemolymph (*T. bielanensis*), or hemo-

lymph of darkling beetle, *Tenebrio molitor* (control), respectively at the mandible underside of a *N. brevicollis* beetle. Of the various behavioral reactions of topically treated ground beetles (10 specimens per tested fluid), disorientation behavior, cleansing behavior, and elytral movements were selected and quantified for 10 min after application of test fluids. The duration of a particular behavior was expressed as a percentage of the observation time with 10 min of video monitoring equal to 100%.

In the same way the percentage of time spent in cleansing behavior and disorientation was measured from mixed groups of carabid beetles (four specimens of *Nebria brevicollis*, three specimens of *Pterostichus oblongopunctatus*) within 10 min after having been topically treated with 1 μl of test fluids of authentic pyridopyrazines. Solutions (0.05, 0.1, and 0.5 M) of 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one, 2,3-dimethoxypyrido[2,3-b]pyrazine, and 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine in DMSO were used.

RESULTS

Origin and Depletion of Droplets of T. bielanensis

After a slight mechanical molestation with forceps, specimens of *T. bielanensis* release sticky whitish droplets from integumental openings called pseudocells (Figure 1A,B). Compared to these pseudocellar fluids, hemolymph samples show a slight violet to whitish coloration. Pseudocellar droplets appear especially at the rear border of the tergites. A careful examination of this springtail species reveals the presence of numerous pseudocells on both its dorsal and ventral sclerites. The total number of dorsal pseudocells is 48, and they are located at antennal bases (three per half) and rear borders of the head (2), thoracic (3 × 2), and abdominal tergites (5 × 2; 1 × 3; Figure 1A,B). The 36 ventral pseudocells are associated with head, coxae, collophore, and abdominal sternites. Numbers and arrangement of pseudocells represent taxonomically important species-specific characters in onychophoran springtails.

The integument and pseudocells of *T. bielanensis* are surrounded by macrotubercles (Figure 2g–i), which are composed of numerous apically fused microtubercles (Figure 2a,b). Pseudocells show an oval shape (diameter about 20 μm; Figure 2a,e) and represent integumental depressions. Externally they are marked by 15–20 longitudinally interrupted ribs that are arranged on the surface of a longitudinally divided cap, also seen from the interior view (Figure 2e). Obviously the external ribs are composed of several fused microtubercles (Figure 2b). SEM examination of shedded old *T. bielanensis* cuticles revealed that the complete pseudocell is molted (deformated caps and surrounding macrotubercles).

Both cap halves and ribs do not survive treatment with 5% potassium

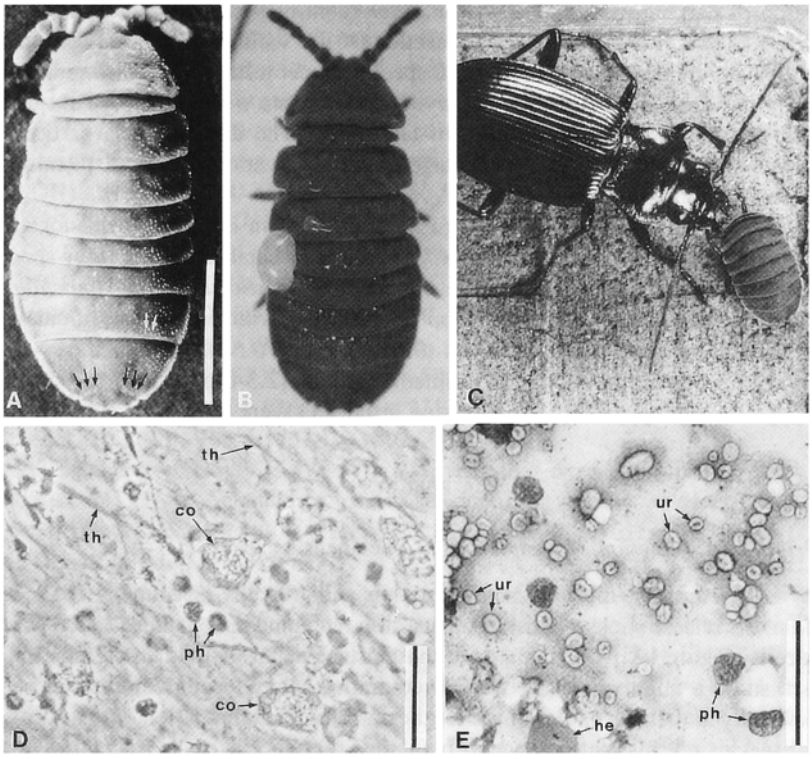

FIG. 1. Position of pseudocells in *T. bielanensis* (arrows) along posterior borders of tergites IV (two pseudocells marked) and V (six pseudocells marked) (A). Depletion of pseudocell secretion, and hemolymph at left posterior border of tergite I (B). Rolling-up behavior of *T. bielanensis* (right) after a slight contact with ground beetle *nebria brevicollis* (left) (C). Microscopic view of pseudocell secretion (D: phase contrast) and hemolymph (E: Wright stain). Abbreviations: co: coagulocytes, th: protein threads; ph: prohemocytes; ur: urate crystals; he: hemocyte. Bars in A B: 2 mm; in D, E: 20 μm.

hydroxide, which also destroys integumental macrotubercles (Figure 2c). Macerated pseudocells only are left as oval-shaped stable rings (Figure 2c–e), which serve as an entrenchment zone for the two cap halves in nonmacerated pseudocells. Specimens of *T. bielanensis* that were killed on Dry Ice exhibited pseudocells with a complete opening sequence (Figure 2a,f–i). At first the two cap halves broke longitudinally (Figure 2f), then pseudocellar fluid was squeezed out and both cap halves were pressed outward (Figure 2g). Later pseudocellar droplets may cover not only the external pseudocell area (Figure 2h) but are

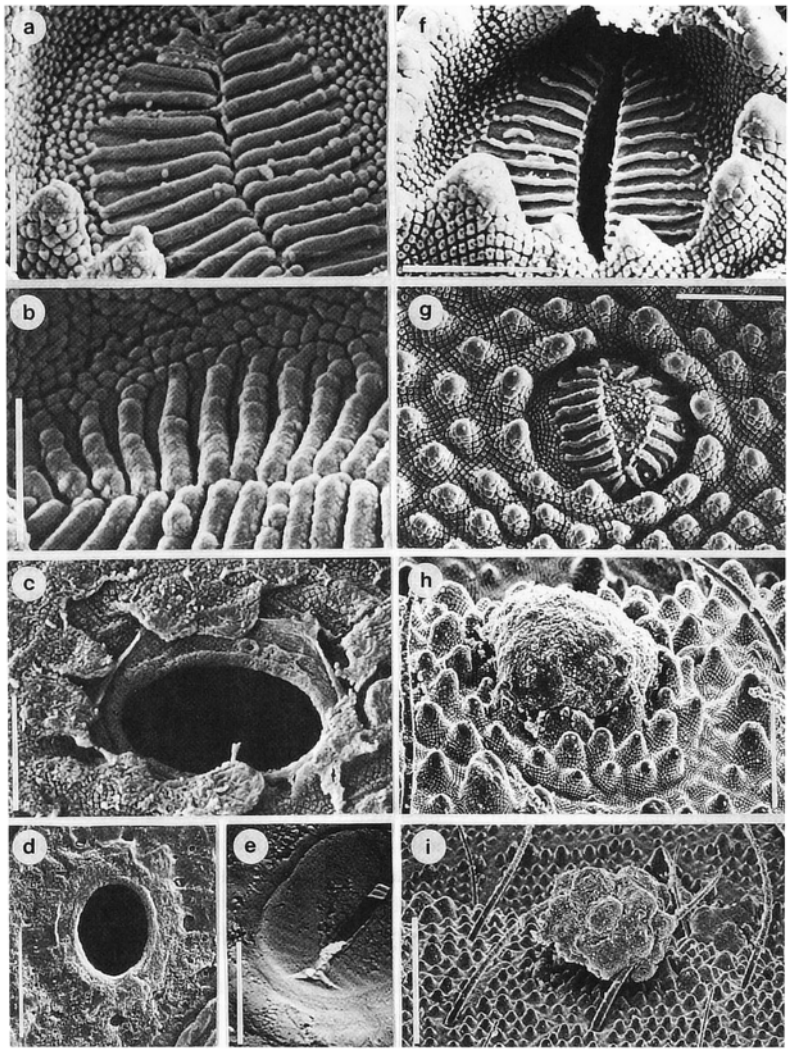

FIG. 2. SEM views of *T. bielanensis* pseudocells (a–i). Oval-shaped pseudocells are externally surrounded by microtubercles which fuse to produce 15–20 longitudinally interrupted ribs arranged on the surface of a longitudinally divided cap (a,b). External (c) and internal (d,e) view of a pseudocell without (e) and after (c,d) treatment with 5% KOH. Macerated pseudocells only remain as oval-shaped stable rings (c,d), which serve as an entrenchment zone for the two cap halves in nonmacerated pseudocells (e). Opening sequence (a,f–i) of a pseudocell (external view) with longitudinally breaking (f) and outward pressing (g) of cap halves and squeezing of pseudocellar secretion (h). Residue of previously squeezed pseudocellar droplets remain adhering at setae and macrotubercles of the collembolan integument (i).

even found side by side along the rear border of a sclerite. The remains of previously squeezed pseudocellar droplets may be found as amorphous material adhering to setae and macrotubercles of the collembolan integument (Figure 2i).

A microscopic investigation of hemolymph samples and pseudocellar fluid shows remarkable differences. Freshly collected pseudocellar fluid contains various prohemocytes (Figure 1D; ph: 7 μm diameter) and several irregular shaped coagulocytes (co: 20 μm diameter), which may quickly burst and thereby release clouded, threadlike filaments (Figure 1D, th). In contrast, hemolymph samples are characterized by many ellipsoid urate crystals (Figure 1E, ur: 2–5 μm diameter), lipid droplets, prohemocytes, and a few hemocytes (he) including coagulocytes. After repeated mechanical molestations, pseudocellar fluid contains urate crystals whereas coagulocytes are almost missing. This observation favors the idea that the composition of initially squeezed pseudocellar fluid differs from hemolymph samples. Only after repeated molestations do pseudocells seemingly exude droplets of hemolymph.

In order to test this hypothesis, peptide patterns of originally squeezed pseudocellar fluids were electrophoretically compared with collophore hemolymph droplets (Figure 3). Native disk PAGE exhibits about six common peptides (51, 68, 230, 240, 430, 770 kDa) in both fluids, whereas several peptides are exclusively present in one type of fluid only (Hemolymph: 85, 125, 190, 285, 290, 350, 510, 630 kDa; pseudocellar fluid: 66, 127, 560, 680 kDa). SDS disk PAGE results in nine common peptides (18, 23, 33, 42, 44, 48, 54, 58, 115). However, pseudocellar fluid (13.5, 16, 25, 68, 70, 77, 127, 135, 145) and hemolymph (22, 38, 39, 65, 73) exclusively contain five to nine peptides, which underlines the different composition of the two collembolan fluids. We conclude, therefore, that the initially emitted pseudocellar fluid represents a secretion distinctly different from hemolymph; removing this secretion with a piece of filter paper and subsequent molestation of the *Tetrodontophora* specimen results in emission of tiny hemolymph droplets through the pseudocellar orifice.

Chemical Constituents

A GC-MS investigation of the pseudocellar fluid revealed the presence of three alkaloids and common C_{16} and C_{18} fatty acids (Figure 4). According to their mass spectra, two of the alkaloids were identified as 2,3-dimethoxypyrido[2,3-b]pyrazine (**1**) and 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine (**2**), both of which had been previously isolated from whole body extracts of *T. bielanensis* (Budesinsky et al., 1986). The third, previously unknown alkaloid, exhibited the mass spectrum shown in Figure 5 with a molecular ion at m/z = 177 (14 mass units less than **1**). A high-resolution mass spectrum proved its composition to be $C_8H_7N_3O_2$.

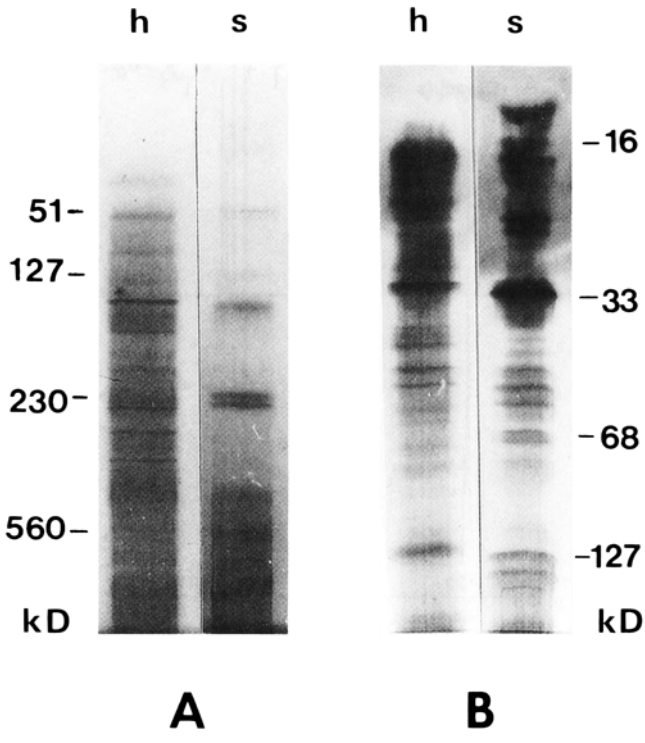

FIG. 3. Native disk PAGE (A) and SDS-PAGE (B) of *Tetrodontophora bielanensis* hemolymph samples (h) and pseudocellar fluid (s). Dilution of each body fluid 1:80 (applied volume 1 μl). Inserted molecular masses of proteins were determined by using high- (A) and low-molecular-weight reference proteins.

Apart from the singlets of the methoxy groups of **1** and **2**, the ^{1}H NMR spectrum of the pseudocellar fluid showed an additional singlet at 4.06 ppm that could also be attributed to a methoxy group. This led to the conclusion that the unknown alkaloid was a demethylated analog of **1**. To elucidate the position of the remaining methoxy group in **3** and to prove its structure, a synthesis of the two possible methoxypyrido[2,3-b]pyrazinones was carried out. According to the method of Anschütz (1906) for the synthesis of imidates, ethyl dichloromethoxyacetate was heated with 2,3-diaminopyridine (**A**) in the presence of triethyl amine in toluene. The product proved to be a 9:1 mixture of the two regioisomers **I** and **II** of methoxypyrido[2,3-b]pyrazinone, of which the major compound **I** was identical with the unknown natural product **3**. Structural assignment of the synthetic products was achieved by use of gated-decoupled ^{13}C

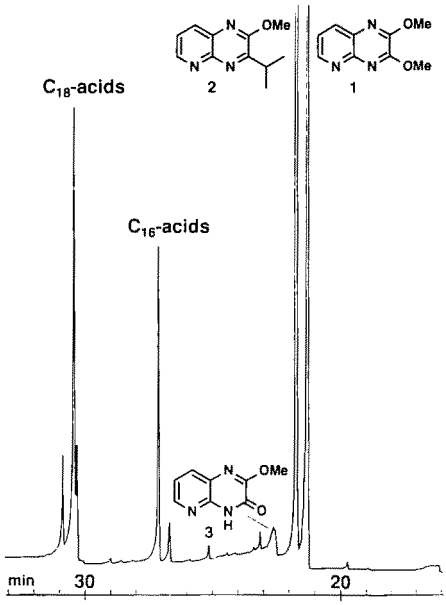

FIG. 4. Gas chromatogram of pseudocellar fluid from *Tetrodontophora bielanensis*, 30-m DB-5, temperature programmed from 60°C to 300°C at 5°C/min, on-column injection.

NMR spectroscopy of *N*-methylated compounds. These derivatives were obtained by reaction of NaH/DMSO/MeI with **I** or 1*H*,4*H*-pyrido[2,3-b]pyrazine-2,3-dione [**B**, obtained by reaction of 2,3-diaminopyridine (**A**) with diethyl oxalate according to the procedure of Weinstock et al. (1981)]. The bridging C atoms are found between 123 and 133 ppm for C-8a and 140 and 150 ppm for C-4a in the ^{13}C NMR spectra of pyrido[2,3-b]pyrazines synthesized by us and other authors (Nagel et al., 1979). The signal for C-4a in the dimethoxy compound **1** shows a quartet with $J_{C,H}$ = 5 and 11 Hz while C-8a is a doublet with $J_{C,H}$ = 8 Hz (see Figure 6A). On the other hand, the C-4a and C-8a signals of 1,4-dimethyl-1*H*,4*H*-pyrido[2,3-b]pyrazine-2,3-dione (**C**) are degenerated to broad multiplets due to coupling to the H-atoms of the *N*-methyl groups (see Figure 6C).

By *N*-methylation of **I** the derivative **D** is obtained, and its spectrum shows signals of C-8a as a doublet with J = 8 Hz and of C-4a a broad multiplet (see Figure 6B). This reveals that C-4a is coupling to the *N*-methyl group, which is thus located at N-4. Therefore, the alkaloid **I** is 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one, the natural alkaloid **3.** This is in accordance with the finding of **I** as the main product in the synthesis, since the more nucleophilic 2-amino

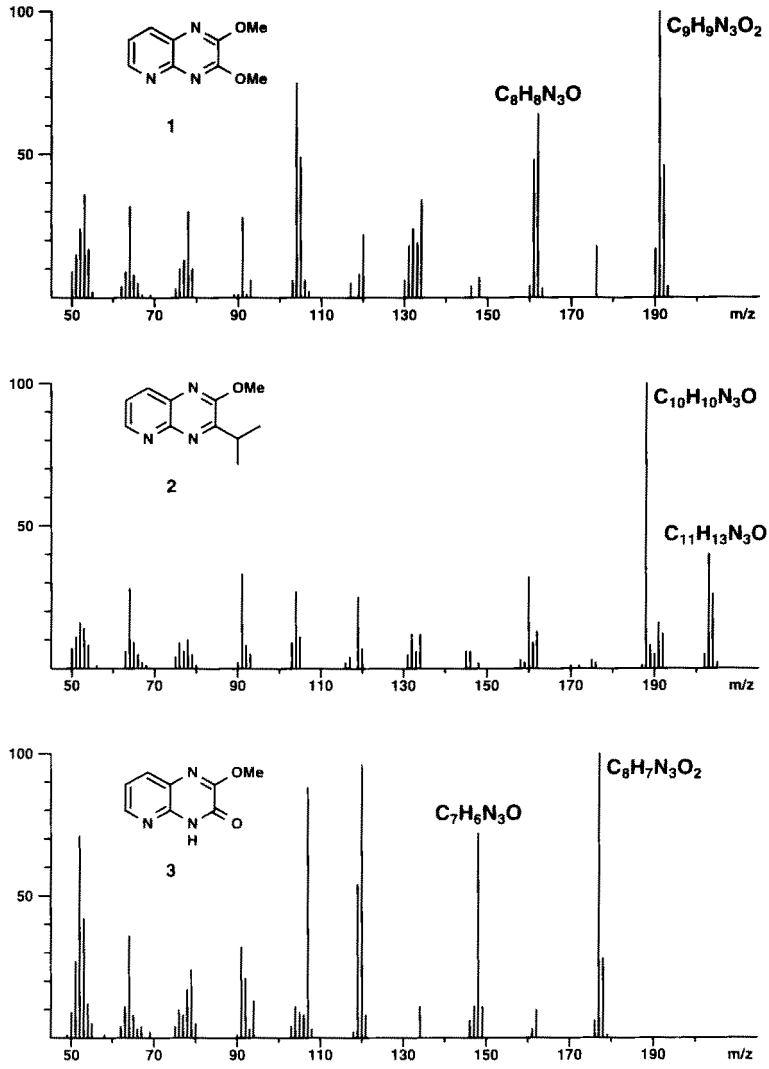

FIG. 5. Mass spectra of the pyridopyrazines **1**, **2**, and **3**.

group of 2,3-diaminopyridine reacts preferentially with the more reactive dichloromethoxymethyl group of the condensing acetate. For bioassays, the two alkaloids **1** and **2** were prepared for the first time starting from 2,3-diaminopyridine (**A**, see Figure 7). Condensation with diethyl oxalate yields the dione **B**, which could not be selectively *O*-methylated using diazomethane, trimethyl-

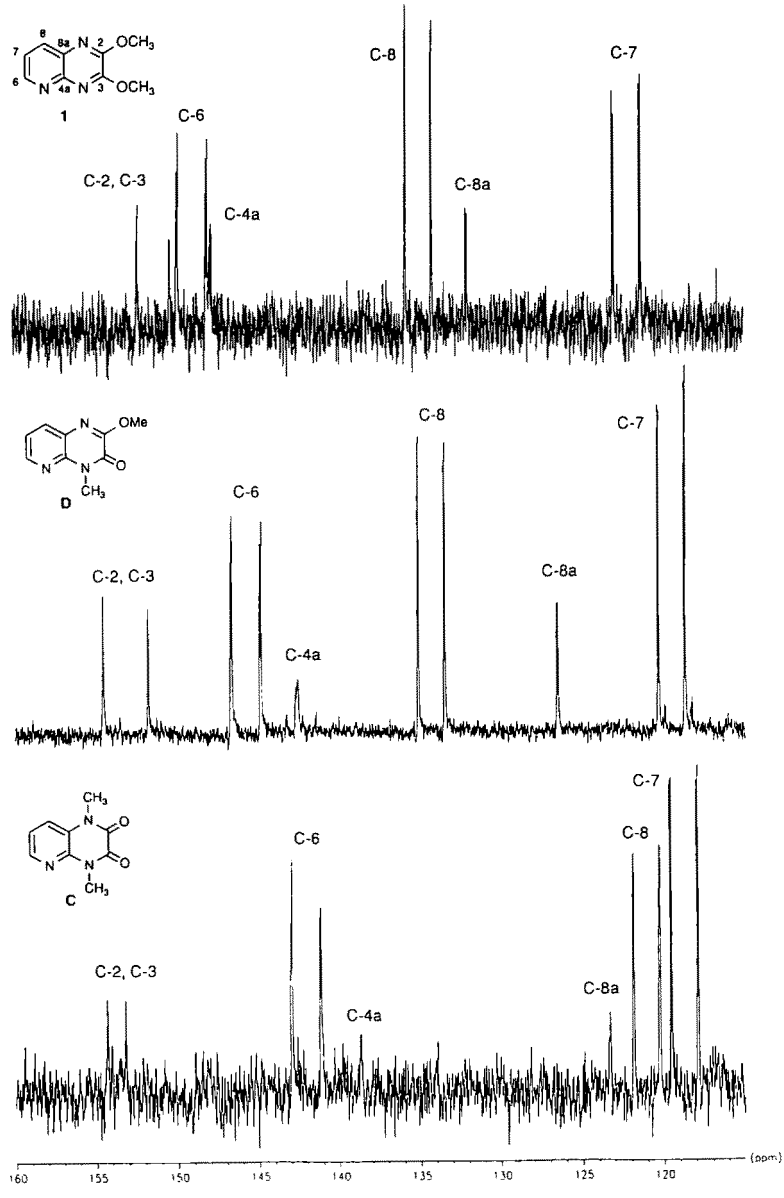

FIG. 6. Gated-decoupled ^{13}C NMR spectra of: 2,3-dimethoxypyrido[2,3-b]pyrazine (**1**), 4-methyl-2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (**D**), and 1,4-dimethyl-1*H*,4*H*-pyrido[2,3-b]pyrazine-2,3-dione (**C**).

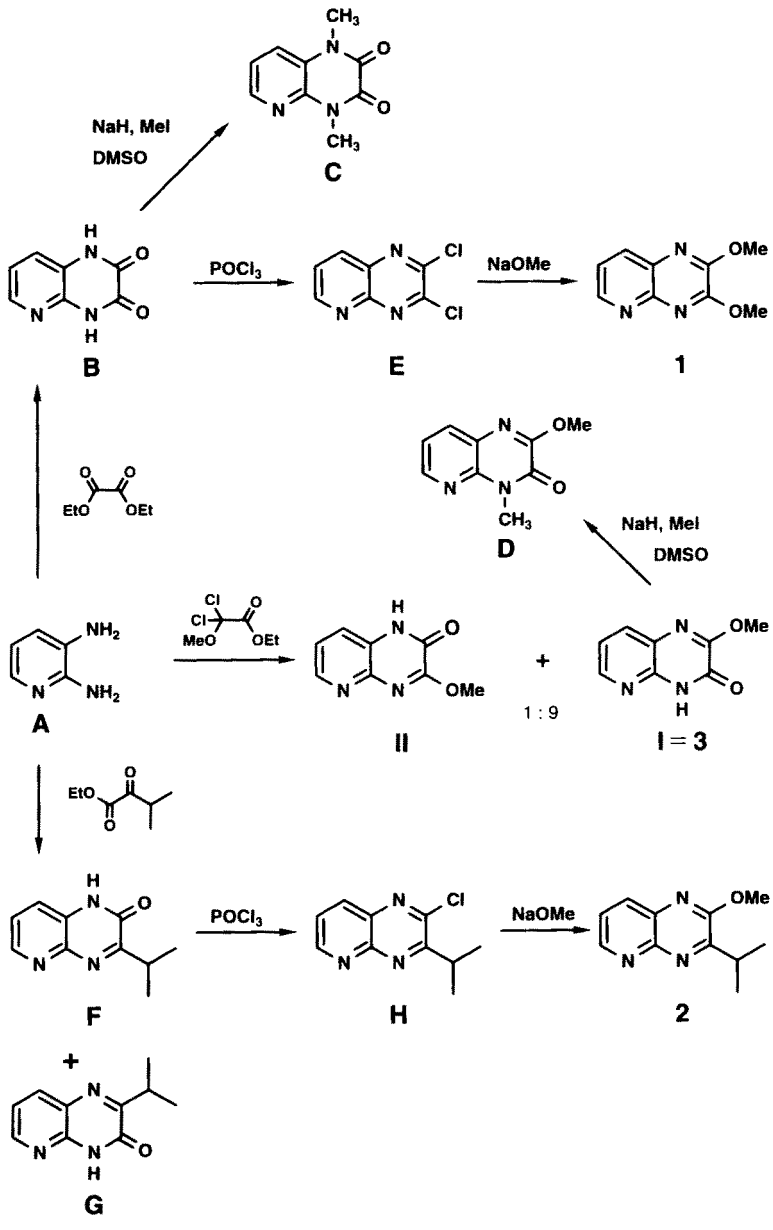

FIG. 7. Synthesis of the pyridopyrazines **1**, **2**, **3**, **C**, and **D**.

oxonium tetrafluoroborate, or Ag_2O/MeI. The results of the methylations are similar to those obtained by Cheeseman in the methylation of hydroxyquinoxalines (Cheeseman, 1955). Instead, chlorination with $POCl_3$ to the dichloride **E** followed by nucleophilic displacement with sodium methoxide yielded the alkaloid **1.** In a similar reaction, methyl 3-methyl-2-oxobutyrate (Weinstock et al., 1981) was condensed with **A** to yield a mixture of 3-isopropyl-1*H*-pyrido[2,3-b]pyrazine-2-one (**F**) and 2-isopropyl-4*H*-pyrido[2,3-b]pyrazine-3-one (**G**), which were separated by column chromatography. Chlorination of **F** to **H** and reaction with sodium methoxide yielded the desired alkaloid **2.** The spectral data of **1** and **2** were identical to the values reported by Budesinsky et al. (1986).

The total concentration of the three pyridopyrazines in adult specimens is about 0.1 mol/liter within pseudocellar fluid and up to 0.05 mol/liter in hemolymph samples. 2,3-Dimethoxypyrido[2,3-b]pyrazine (**1**), 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine (**2**), and 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (**3**) could be found within pseudocellar fluids and hemolymph samples of both males and females of *T. bielanensis* (Table 1). Pseudocellar secretions of the springtail larvae contain approximately equal amounts of **1** and **2;** the 3-isopropyl-2-methoxy-derivative represents the only pyridopyrazine of the larval hemolymph. Minor amounts of **1** and **2** are also present in the guts of both sexes, the ovaries and the male fat body; however, neither feces nor food of *T. bielanensis* contain even traces of these alkaloids.

Biological Significance of Reflex Bleeding

The target animals, *Nebria brevicollis*, are nocturnal polyphagous carabids that clearly prefer springtails as prey (Pollet and Desender, 1988). When there was a contact of *T. bielanensis* with antennae of the carabid beetle, the beetle's forelegs jerked forward and the springtail showed curling behavior (Figure 1C). If the beetle tried to grasp the springtail with its mandibles, *T. bielanensis* exuded a droplet from a pseudocell. Aggressive behavior of *Nebria* was immediately interrupted, and the carabid beetle tried to clean its mandibles. In cleansing behavior the mandibles are spread out and dug into the ground (Carrel and Eisner, 1974). The beetles further showed uncoordinated movements, fell on their back (disorientation), and usually secreted a clear fluid from their mouth. After a contact with pseudocellar fluid, the beetles usually extended their legs, slightly lifted their abdomens, and spread their elytra (elytra movement). A second attack of the carabid beetle was never observed.

Topical treatment of carabid mandibles and quantitative analyses of subsequent disorientation, cleansing behavior, and elytral movement revealed distinctly different results depending on the kind of fluids used in the experiment (Figure 8). Cleansing behavior was observed with all three test fluids but was only significantly increased ($P < 0.01$, nonparametric STP test; Sokal and

TABLE 1. PERCENTAGE OF PYRIDOPYRAZINES IN VARIOUS BODY COMPARTMENTS OF *T. bielanensis*

	Percentage		
	2,3-Dimethoxy-pyrido[2,3-b]-pyrazine (**1**)	3-Isopropyl-2-methoxy-pyrido[2,3-b]-pyrazine (**2**)	2-Methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (**3**)
Female			
Pseudocellar fluid	23.5	39.0	37.5
Hemolymph	48.7	18.3	33.0
Gut	x	x	–
Fat body	–	–	–
Ovaries	x	x	–
Male			
Pseudocellar fluid	25.5	31.5	43.0
Hemolymph	65.2	28.8	6.0
Gut	x	x	–
Fat body	x	x	–
Larva			
Pseudocellar fluid	42.6	57.4	–
Hemolymph	–	x	–
Feces (adults	–	–	–
Food (wood particles)	–	–	–
Food (granulated pollen)	–	–	–

[a] x: present in small amounts, but not quantified; –: absent.

Rohlf, 1981) if pseudocellar fluid had been topically applied previously on mandibles of the ground beetle. Disorientation of carabid beetles resulted after contact with pseudocellar fluid only, whereas elytral movement was observed exclusively after testing of pseudocellar fluid and *T. bielanensis* hemolymph (Figure 8). Distinct cleansing behavior was especially released after a topical application of 1 μl of 0.5 M DMSO solutions of **3** and **1** and even a 0.1 M DMSO solution of **2**. As compared to the DMSO solvent, which did not harm the beetles, strong disorientation could be observed only if 0.5, 0.1, and 0.05 M solutions of **2** were applied. Both cleansing behavior and disorientation of carabids increased with increasing concentrations of the three authentic pyridopyrazines (see Discussion). The pronounced activity of **2** (Table 1) is interesting in three respects: (1) Compound **2** quantitatively represents the main pyrazine in pseudocellar fluids and hemolymph of males and females of *T. bielanensis*. (2) It constitutes the only pyridopyrazine in the hemolymph samples

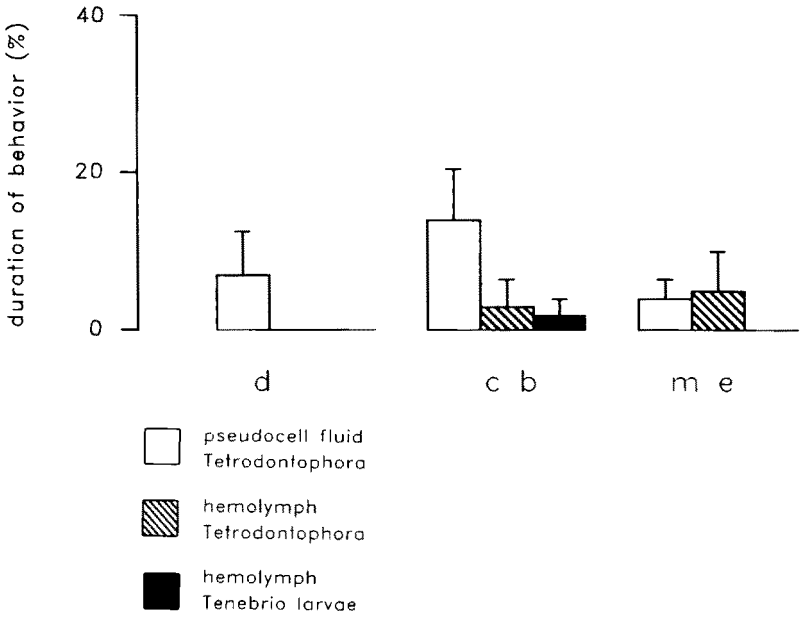

FIG. 8. Percentage duration of disorientation (d), cleansing behavior (cb), and elytral movements (me) of carabid beetles *Nebria brevicollis* within 10 min after having been topically treated with 1 μl test fluids. Pseudocellar fluid (light bar) and hemolymph of *T. bielanensis* (shaded bar) as well as hemolymph of *T. molitor* (black bar; controls) were used for tests. Ten carabid beetle specimens were used per test fluid (95% confidence values are denoted). Hemolymph samples of both *Tetrodontophora* and *Tenebrio* caused no disorientation behavior.

of the *Tetrodontophora* larvae. (3) The total concentration of the three pyridopyrazines in adult specimens amounts to about 0.1 mol/liter within pseudocellar fluids and up to 0.05 mol/liter in hemolymph samples.

DISCUSSION

Origin and Depletion of Pseudocellar Droplets of T. bielanensis

Several springtail species devoid of pseudocells show reflex bleeding behavior after nonspecific irritations such as mechanical contact or vibration (Paclt, 1956). Reflex bleeding at soft integumental areas usually is connected to rolling-up behavior and seems to be due to an increase in internal body pressure. Pseudocells are seemingly restricted to Tullbergiinae, and Pachytullbergiinae, Onychiurinae, and Tetrodontophorinae and probably represent an

advanced condition as compared to reflex bleeding. The primitive type of pseudocells is externally located at the trochanters of legs of *Onychiurus* species and is not distinctly differentiated from the surrounding integument. Furthermore, it is considerably smaller and shows no circular ring as observed in *T. bielanensis* (Rusek, 1984). The normal advanced type of pseudocell, however, is enlarged and corresponds to pseudocells of *T. bielanensis* or *Onychiurus armatus* (Rusek and Weyda, 1981). Depending on the species, the number, distribution, and external gross and fine structure of advanced springtail pseudocells may vary considerably (Hale and Smith, 1966; Rusek and Weyda, 1981). Morphologically there is a remarkable congruency between pseudocells of *T. bielanensis* and *Onychiurus armatus* that becomes evident after SEM and TEM studies (this paper; Rusek and Weyda, 1981). Pseudocellar caps are epicuticular structures that do not survive KOH maceration. On the contrary, the KOH-resistant stable pseudocellar ring represents a part of the endocuticle. One to several secretory cells are distinctly associated with the pseudocell. These cells adhere at the interior border of pseudocellar caps and are distally floating within hemolymph. The secretory cells of *O. armatus* contain many voluminous secretory vesicles (Rusek and Weyda, 1981), which clearly indicates that pseudocells are secretory structures par excellence. After slight molestation, rupture of the epicuticular caps occurs, which simultaneously destroys the secretory cells and liberates their contents. Subsequent irritations result in liberation of hemolymph (reflex bleeding) through the pseudocellar ring. Whether the closure of the longitudinal pseudocellar slit after molestation is due to elasticity of certain structures of the pseudocell is not yet clear. However, the suggestion of Koncek (1924), that secretory cells under the pseudocell would be brushed aside during its opening and hemolymph-outflow may be rejected.

As was shown by native disk PAGE, hemolymph samples contain more exclusive peptides as compared to pseudocellar secretion. However, SDS-PAGE produced more exclusive peptides in the pseudocellar secretion as compared to hemolymph samples. Due to the decreased pore diameter of PhastGel gradient 8-25 after SDS treatment, high-molecular-weight plasma proteins probably cannot diffuse into the gel, which may be responsible for this effect. Special peptides of pseudocellar secretions probably originate primarily from pseudocell coagulocytes and may principally represent clotting proteins, toxic peptides, or enzymes involved in pyridopyrazine biosynthesis. However, clotting proteins should be also present in hemolymph samples because coagulocytes are also present. Moreover, bioassays with authentic pyridopyrazines produced similar effects as the crude pseudocellar secretion. Therefore, the presence of toxic proteins within the pseudocellar coagulocytes seems unlikely.

The distribution of the three pyridopyrazines in different compartments of *T. bielanensis* (Table 1) cannot reveal structures or tissues where these unusual compounds are synthesized. However, it seems possible that pyridopyrazines are

synthesized within secretory cells located below the pseudocells. From there pyridopyrazines might be transfered into hemolymph and subsequently distributed interiorly within the body of *T. bielanensis*. Three arguments would favor this suggestion: As compared to hemolymph, the highest total amounts of pyridopyrazines are found in pseudocellar fluids (1). The complete series of pyridopyrazines is only present in the pseudocellar fluid, not in other compartments of larvae or adults (2). If the 3-isopropyl-2-methoxy-derivative **2** should represent the precursor for **1** and **3,** it is remarkable that the percentage of **2** decreases from pseudocellar fluid to hemolymph (3, Table 1). On the other hand, it may not be excluded that one or two pyridopyrazines might also be synthesized somewhere in the interior body of the springtail and then subsequently enriched within the pseudocellar secretory cells.

Biologically Active Constituents

Budesinsky et al. (1986) isolated **1** and **2** from total body extracts of 7300 *T. bielanensis*. Both pyridopyrazines and the newly identified compound **3** have been isolated for the first time from their natural glandular sources. Comparable nitrogen-containing aromatic heterocycles from arthropods contain either fewer [e.g., methyl-8-hydroxyquinoline-2-carboxylate; 1-methyl-2-quinolone; glomerin; homoglomerin (Dettner 1987, Numata and Ibuka, 1987)] or more nitrogen atoms [various pteridines (Numata and Ibuka, 1987)]. The biosynthetic pathway leading to the pyridopyrazines is unknown. Our HPLC analyses of body extracts of males and females of *T. bielanensis* showed that this species contained considerable amounts of uric acid, the granules of which are also found in hemolymph samples. This is reasonable since Collembola have no Malpighian tubules as excretory organs (Feustel, 1958). Uric acid therefore, might be an available pool for nitrogen-containing compounds in all collembolan species.

Intermediates for the biosynthesis of pyridopyrazines are unknown, although purines (and uric acid) might serve as precursors for related pteridines. Nevertheless, we regard **2** to be the precursor of the other two pyridopyrazines. An oxidative degradation of the isopropyl side chain would produce **3,** which could be methylated to produce **1.** This pathway is supported in three respects: The pyridopyrazine **2** is the only compound of this type present in the larval hemolymph, which supports the suggestion that larvae may exhibit more primitive characteristics than the adults (Table 1). At the same time this compound shows the maximal deterrent activity as compared to the two other analogs (Figure 9). In addition, **3** as the final product of the biogenetic sequence could not be detected in various body compartments (Table 1). A precursor for the pyridopyrazine **2** might be valine or its amide, as was suggested for the biosynthesis of 3-alkyl-2,6-dimethylpyrazines in ants (Numata and Ibuka, 1987).

Synthetic derivatives of pyrido[2,3-b]pyrazines, which have also been named 1,4,5-triazanaphthalenes or 5-azaquinoxalines, are well known as anti-

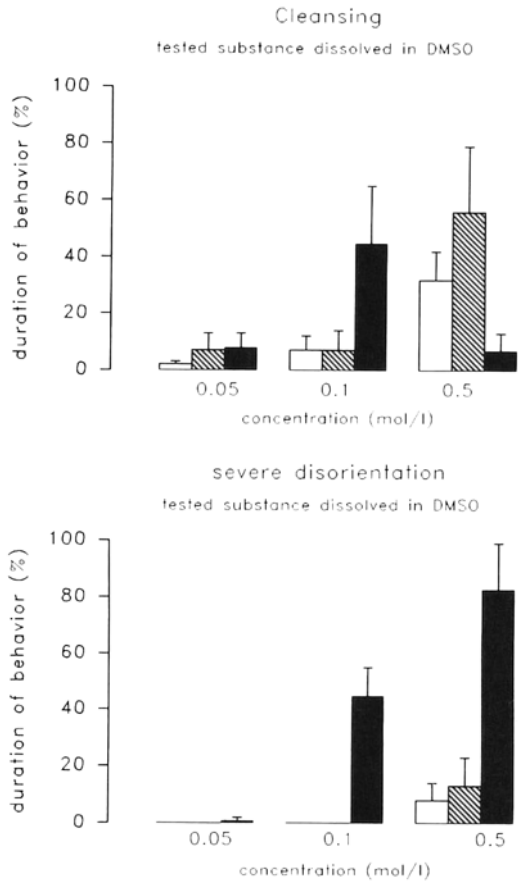

FIG. 9. Percentages duration of cleansing behavior (above) and disorientation (below) of mixed groups of carabid beetles (*Nebria brevicollis*, *Pterostichus oblongopunctatus*) within 10 min after having been topically treated with 1 μl of test fluids. Test fluids were 0.05, 0.1, and 0.5 M solutions of authentic 2-methoxy-4*H*-pyrido[2,3-b]pyrazine-3-one (light bar), 2,3-dimethoxypyrido[2,3-b]pyrazine (shaded bar), and 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine (black bar) dissolved in DMSO. With each test fluid seven carabid beetles (four specimens of *N. b.* and three specimens of *P. o.*) were used (95% confidence values are noted).

metabolites and folic acid antagonists. Whereas various 8-substituted amino derivatives of pyrido[2,3-b]pyrazines show antibacterial activities, a number of 3,6-diamino-derivatives exhibits antiviral, diuretic, and antiinflammatory properties (Cheeseman and Cookson, 1979). Our results clearly show that these unique pyrazines are not directly derived from food. They must be synthesized

within internal tissues of Collembola. The distribution pattern may indicate that coagulocytes contribute to the complete set of pyridopyrazines present, because **3** is exclusively found within this compartment.

Biological Significance of Reflex Bleeding

By comparing results from Figure 9, it is evident that biological activity of the three pyridopyrazines increases with lipophilicity. The significant decrease of cleansing behavior after application of a 1-μl droplet of a 0.5 M solution of 3-isopropyl-2-methoxypyrido[2,3-b]pyrazine on a beetle mandible is a consequence of the fact that these beetles were disorientated during almost the whole observation period. Cleansing behavior, which was expected to be increase after treatment with a 0.5 M solution, decreased at the expense of a strong disorientation behavior. Another preliminary experiment demonstrated that the three compounds with different biological activities might also act synergistically in the laboratory and in the field. When 1 μl of a DMSO solution (0.1 M in total pyridopyrazines: mixture of **1, 2,** and **3** in a ratio of 3 : 3 : 1) was applied topically to a mandible of a carabid beetle, the duration of cleansing behavior and disorientation was comparable to 1 μl of a pure 0.1 M solution of the most active compound **2.** This synergism indicates that compounds **1** and **3,** with weaker activities when tested alone, exhibit increased biological activities when tested together with **2.**

Many possibilities exist for springtails to avoid contacts with predators or to escape after being discovered. Primary defenses may include slow movements or cryptic colorations (Bauer and Pfeifer, 1991). Secondary defenses of springtails are their jumping ability, the presence of integumental hairs and scales, and a fast unpredictable stampede (Bauer and Pfeifer, 1991). In the first case, after a mechanical stimulation, Collembola escape from a predator by jumping away within a few milliseconds. The second defensive mechanism is also effective, since such scales and hairs (present in only few families of Collembola) are easily removed and are always found sticking to the mouth parts of arthropods that feed on springtails (Bauer and Pfeifer, 1991).

Concerning the secondary defenses, especially chemical defenses, all important mechanisms as observed in other insects are also found in springtail species. Southeast Asian and Pacific species of the genera *Piroides* and *Corynephora* (Dicyrtomidae) show warning coloration and have a clublike defensive gland located dorsally at the abdominal tip (Greenslade and Suhardjone, 1992; Bauer, personal communication). Many springtail species, especially the eyeless soil-inhabiting Onychiuridae, do not have jumping ability and instead may release defensive compounds from pseudocells or toxic hemolymph chemicals by reflex bleeding (Paclt, 1956). It is probable that these insects may represent a rich source of biologically active new defensive compounds. This is supported by

observations of Weinreich (1968) and Bauer and Pfeifer (1991), who observed hunting behavior of the springtail-feeding rove beetle, *Stenus comma*, which has a protrusible labium to catch collembolan prey. This visually orienting beetle ignores certain collembolan species of a genera *Folsomia*, *Xenyella*, and *Tomocerus* and never feeds on *Onychiurus armatus* or *Podura aquatica*. Since *Podura aquatica* neither possesses pseudocells nor shows reflex bleeding, the presence of internal toxins or epicuticular deterrents might be suggested. Specimens of *Podura aquatica* were caught in 75% of all attacks by *Stenus comma*, but 88% of the seized collembolans were released within a few seconds and about 50% of them survived the attack (Bauer and Pfeifer, 1991). After these interactions, the beetles cleaned their mouthparts and rubbed them in the soil.

In other Collembola the use of alarm pheromones, which may act even across families, has been described (Purrington et al., 1991). The chemical nature of these alarm compounds has not been elucidated, but pseudocellar compounds and hemolymph toxins such as the pyridopyrazines may play a role in alarm behavior. The smell of *Tetrodontophora bielanensis* strongly resembles the smell of toxic coccinellid beetles that exhibit reflex bleeding (unpublished observation). Some 2-methoxy-3-alkylpyrazines have been described as warning odors (Moore et al., 1990; Dettner and Liepert, 1994). Whether *T. bielanensis* contains similar odor signals remains to be clarified, but synthetic alkaloids **1–3** are almost odorless.

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Book Review

Bombardier Beetles and Fever Trees, A Close-Up Look at Chemical Warfare and Signals in Animals and Plants. William Agosta. New York: Helix Books, Addison-Wesley Publishing Company, 1996. US $25, Canada $34. ISBN 0-201-62658-6

This is a very entertaining and excellent introduction to the broad field of chemical ecology. Although the author does not include a preface in which he might have told us to whom he was writing, it appears that the book is written for a general audience. The word "pheromone" occurs in the book, but such words as "semiochemical," "allomone," and "kairomone" are not used. Professional scientists working in chemical ecology will find it interesting reading and, as I did, will probably find that it broadens one's perspective on the range of interactions in chemical ecology. The book does not go into experimental details or theory in chemical ecology, and there is no emphasis on chemistry, except to mention some chemicals by name. Examples are chosen from many groups, including plankton, bacteria, fungi, higher plants, nematodes, insects, and a variety of vertebrates, including mammals and humans to illustrate diversity in chemical ecology. The book contains nine chapters, and 16 black and white half-tone illustrations. The author concludes the book with a short "Note to the Reader" explaining the basis of scientific classification and units of measurement. There is an index.

Chapter 1, "Nature's Storehouse of Chemicals," introduces the universality of chemicals and chemical ecology in the lives of all organisms. The author attempts to dispel the phobia that some have of anything chemical by offering examples of beneficial chemicals in our foods that may provide anticancer protection, and vitamins that are essential for good health.

Chapter 2, "Chemical Defenses in the Plant World," provides interesting examples of the many plant–animal, particularly plant–insect, interactions with examples drawn from the *Diabrotica*–curcurbitacin story, *Larrea* spp. (creosote bushes), and some insects that feed with impunity upon them, light-activated plant defensive chemicals such as hypericin, and plant-produced compounds such as paper factor, phytoecdysones, and precocenes, that alter hormonal control of growth and development in insects. The chapter ends with a section entitled "Can Plants Call for Help?" with examples of plant–plant interactions induced by various insect and mite attacks.

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Chapter 3, "How Animals Use Chemical Warfare," deals mainly with defensive chemical ecology, including toxic chemicals in the cuticle of insects and skin of frogs, special glandular secretions, and nematocysts of cnidarians. The interesting, but little understood, process of self-anointing common in some vertebrates is described. As the title of the book promises, the story of bombardier beetles that can aim and spray a hot mix of chemicals at an attacker receives good coverage. One section deals with how humans cope with some natural toxins, including avoiding the puffer fish toxin when it is used as food, and important uses of its toxin in neurophysiological studies. Agosta points out that tetrodotoxin is not produced by the fish, but comes from bacteria in the fish. The author admonishes us that we need to protect our natural environmental resources, which are likely to hold many yet to be discovered treasures.

Chapter 4, "Warfare in Simple Organisms," deals with bacteria and fungi, and examples show how these are important to agriculture and human medicine. Penicillin, aflatoxin, BT pesticides, avermectin and its slightly modified derivative, ivermectin, are described. Merck's development of avermectin and ivermectin, and the potential for these in management of river blindness in several parts of the world make a fascinating story.

Chapter 5, "Medicines and Drugs from Plants," introduces folk medicines and drugs that we get directly from plants or from derivatives of plant chemicals, such as aspirin. The fever tree referred to in the book title is the Peruvian *Chinchona* tree that yields quinine; the scourge of malaria and its treatment with quinine and derived drugs are discussed at some length. This chapter contains a few pages on mood-altering drugs, such as those from coca plants, peyote plants, psilocybin from certain mushrooms, and cannabis, and a page or two on the possible use of healing plants by chimpanzees. The author ends this chapter with emphasis on how important it is to preserve the great diversity of plants and other organisms that still exist, but which we are losing very rapidly at present, and to investigate them for substances useful to humans.

Chapter 6, "Chemicals and Lifestyles," describes how chemicals play a vital role in the life of all organisms, with examples drawn from fireflies, fish, and other organisms with antifreeze in their systems; natural adhesives produced by numerous organisms; and products such as silk. Several pages are devoted to discussion of camouflage and warning colors, display colors for attracting mates, and mimicry.

Chapter 7, "Chemical Messages within the Family," describes the early demonstration of a moth pheromone by Fabre, who attracted numerous male moths to his open window with a caged female emperor moth, and the first pheromone identified, bombykol, by Adolph Butenandt. Other pheromonal roles are explored, including pheromones in vertebrates and the vertebrate vomeronasal system involved in detecting pheromonal chemicals. The author discusses possible pheromones in plants, some practical applications of pheromones, and

concludes the chapter with a brief presentation of documented and potential human pheromones.

Chapter 8, "Chemical Messages to the Wider World," concerns messages between hunters and their prey. It was fascinating to learn that some fungi secrete attractants that lure nematodes and perhaps other small organisms into entangling sticky filaments, where they will be digested as food for the fungus. Human applications of chemical ecology in pest control and in breeding parasite- and disease-resistant plants are given.

Chapter 9, "Making use of Nature's Molecules," reinforces Chapter 1 by summarizing the universal occurrence of chemicals in the life of all living organisms and presents a challenge to scientists to discover the nature of these chemicals and the role(s) they play in the life of those organisms. The author points out that ". . . scientists have searched for chemicals in only a few thousand of the approximately 1,400,000 known living species." Perhaps several million species and their chemical ecology relationships still wait to be discovered. Some known groups of organisms have barely been touched, and Agosta promises surprises and interesting stories for many generations of scientists, if we carefully manage and preserve what we still have. The potential for current gene technology to aid in the search for useful products from the natural world is briefly mentioned in this very short chapter.

Bombardier Beetles and Fever Trees has a handsome jacket cover, is attractively printed, and is written in a lively style. Its modest price makes this 224-page book available to a wide audience, and it will provide entertaining reading for amateur naturalists and professionals alike. It will be a useful book to introduce chemical ecology and nature's diversity to undergraduate students interested in biology, environmental issues, nature, and conservation. It should be available in high school, college, and university libraries. Readers of papers in the Journal of Chemical Ecology will find it light, but entertaining and lively reading.

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PHORETIC EGG PARASITOID, *Telenomus euproctidis* (HYMENOPTERA: SCELIONIDAE), USES SEX PHEROMONE OF TUSSOCK MOTH *Euproctis taiwana* (LEPIDOPTERA: LYMANTRIIDAE) AS A KAIROMONE

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Abstract—The phoretic egg parasitoid, *Telenomus euproctidis* Wilcox (Hymenoptera: Scelionidae) was found more frequently on virgin than on mated female moths of *Euproctis taiwana* (Shiraki) (Lepidoptera: Lymantriidae), when virgin and mated moths were exposed concurrently in the field for 24 hr. A synthetic component of the moth's sex pheromone, (Z)-16-methyl-9-heptadecenyl isobutyrate, attracted both the wasp, *T. euproctidis*, and male *E. taiwana*. These findings suggest that *T. euproctidis* uses the sex pheromone of the female moth, *E. taiwana*, as a kairomone to locate a host female moth and through her the host eggs.

Key Words—*Telenomus euproctidis*, kairomone, sex pheromone, phoresy, *Euproctis taiwana*.

INTRODUCTION

Several species of *Telenomus* egg parasitoids (Hymenoptera: Scelionidae) have developed a special phoretic relationship with the adults of their host species (Clausen, 1972, 1976). Phoresy has been observed in *T. euproctidis* Wilcox (=*Telenomus* sp. cf. *euproctidis*), a parasitoid of the eggs of the tussock moth, *Euproctis taiwana* (Lepidoptera: Lymantriidae), in Okinawa, Japan (Arakaki, 1990). All of the observed female parasitoids were associated with the long anal tufts of adult female moths. As soon as the moth began to lay the first egg-

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mass, the parasitoids left the moth and attacked the host eggs. However, the question of how this parasitoid locates its phoretic host remains unsolved.

It is known that several larval parasitoids use herbivore-induced plant odors associated with their hosts as olfactory cues to locate host larvae (e.g., McCall et al., 1993; Mattiacci et al., 1994; Turlings et al., 1990). However, egg parasitoids basically have a problem in locating their host because (1) the host egg does not feed and, therefore, does not yield herbivore-induced plant odors and (2) noninduced plant odors are not a reliable indicator of host presence. Thus, information that is present for many other parasitoids is not available to egg parasitoids. One possibility is that egg parasitoids might rely upon detectable information from virgin host females, the mothers of their future hosts.

Some nonphoretic egg parasitoids are known to use the female sex pheromones of several lepidopterous species in location of eggs that serve as their hosts (Lewis, 1982; Nordlund et al., 1983; Noldus and van Lenteren, 1985). There has been only one report that the phoretic egg parasitoid, *Telenomus* n. sp., uses a host pheromone in long-range host location (Aldrich et al., 1984). The phoretic association of *T. euproctidis* with *E. taiwana* female moths whose eggs are parasitized by the parasitoid suggests that sex pheromone might be the cue used by the parasitoid to locate the adult female.

Two compounds have been identified as sex pheromone components in *E. taiwana*. These compounds are (*Z*)-16-methyl-9-heptadecenyl isobutyrate (16Me-Z9-17:iBu) and 16-methylheptadecyl isobutyrate (16Me-17:iBu) (Yasuda et al., 1995). In this paper, we test a hypothesis that the phoretic egg parasitoid, *T. euproctidis*, uses the moth sex pheromone as a primary cue for host location.

METHODS AND MATERIALS

Insects. The moths we used in the study originated from individuals collected from a wild population in a cabbage field at Naha, Okinawa, Japan, in 1994. They were maintained for about eight generations in the laboratory at 25 ± 0.5°C under a 16:8-hr light–dark photoperiod. Larvae were reared on a commercially available artificial diet for some lepidopterous larvae (Insecta LF; Nihon Nosan Kogyo Co. Ltd., Kanagawa, Japan). In order to reduce allergic reactions in those working with this moth, we removed the urticating bristles attached to the cocoons by dissolving the cocoons in 1% sodium hypochloride solution for about 10 min and then washing the pupae in tap water.

Attraction and Phoresy of Parasitoids on Intact Virgin and Mated Female Moths. Field experiments were conducted at Okinawa Prefectural Agricultural Experiment Station, Naha, Okinawa, in May 1994. Treatments consisted of either plastic cups containing a single virgin female ($N = 8$ replicates), or plastic cups containing a single 3-day-old mated female (=control: $N = 8$ replicates).

Each 120-ml cup with a moth had 2.5- × 1.5-cm windows cut at opposite sides, and the windows were covered with 3-mm mesh plastic netting. The netting allowed the moth's pheromone to diffuse into the air surrounding the cup. The mesh size also allowed attracted parasitoids to enter the cup. These cups with moths were then placed on the branches of hedge plants such as hibiscus at 5-m intervals, 1.2 m above the ground at around 11:00 hr. After a 24-hr exposure in the field, the cups with moths were placed in plastic bags, one cup to a bag, returned to the laboratory, and placed in a freezer for 1 hr to kill the moth and any parasitoids. The anal tufts of the moths were inspected for parasitoids by using a dissecting microscope, and the parasitoids per moth were counted and recorded.

Response of Parasitoid to Synthetic Sex Pheromone. Five treatments using different components or mixtures of components of a synthetic pheromone for *E. taiwana* and a control were placed in the field at Okinawa Prefectural Agricultural Experiment Station.

The dose and blend of synthetic pheromone components for attraction of *E. taiwana* were determined in a previous experiment (Yasuda et al., 1995). The treatments consisted of (1) 300 μg of 16Me-Z9-17:iBu, (2) 100 μg of 16Me-17:iBu, and (3) a blend of 300 μg of 16-Me-Z9-17:iBu and 100 μg of 16-Me-17:iBu. Chemicals were dissolved in hexane, and the solution was added to a 2- × 5-cm piece of filter paper (Toyo No. 2). The filter paper was then placed 3 cm above a sticky plate in a trap (SE type; Sankei Chemical Co., Kagosima, Japan). Controls consisted of a piece of filter paper treated with solvent only, and three caged virgin *E. taiwana* female (1–3 days old), which were placed in a sticky trap. Treatments were replicated three times (=3 traps per treatment), and the traps were set out at 5-m intervals, 1 m above the ground.

In trial 1 the traps were set out about 18:00 hr on April 24 and collected on May 4, 1995, but baits and trap locations were not changed. The second experiment was conducted from May 22 to 24, during which time the baits and sticky plates were changed to fresh ones at about 18:00 hr each day. Trap locations were exchanged systematically to cancel the possible effect of trap position on trap catches. The numbers of *E. taiwana* males and the numbers and sex of *T. euproctidis* caught on the sticky plates were counted with the aid of a dissecting microscope.

RESULTS

Attraction and Phoresy of Parasitoids on Intact Virgin and Mated Female Moths. A total of 36 *T. euproctidis* were found in the anal tuft of virgin females, but only six were found on the mated females (Table 1). This difference was

TABLE 1. COMPARISON OF ATTRACTIVENESS BETWEEN ONE CAGED VIRGIN AND ONE MATED FEMALE TUSSOCK MOTH, *E. taiwana*, TO FIELD POPULATION OF PHORETIC EGG PARASITOID, *T. euproctidis*. (OKINAWA, MAY 1994) ($N = 8$)[a]

Replicate no.	No. of egg parasitoids found	
	Virgin female	Mated female
1	13	2
2	5	1
3	0	1
4	4	0
5	6	0
6	0	0
7	2	1
8	6	1
Total	36	6

[a] $P < 0.05$ Wilcoxon's signed-rank test.

statistically significant ($P < 0.05$, Wilcoxon's signed-rank test). All parasitoids found on the moths were female.

Response of Parasitoids to Synthetic Sex Pheromone. Large numbers of *T. euproctidis* were caught in traps baited with synthetic sex pheromone for *E. taiwana*, 16Me-Z9-17:iBu alone, and in the blend of 16Me-Z9-17:iBu with 16Me-17:iBu, but significantly fewer were attracted to 16Me-17:iBu alone and to a blank trap (Figure 1). Traps baited with virgin female *E. taiwana* also captured a large number of *T. euproctidis*. All the captured parasitoids were female. Similar results were observed in the total number of *E. taiwana* males captured; many *E. taiwana* males were caught with 16Me-Z9-17:iBu alone, with the blend tested, and with virgin females, but none was caught with 16Me-17:iBu alone or a blank trap. The total number of parasitoids caught was greater in trial 1 than in trial 2.

DISCUSSION

Some species of egg parasitoids are known to use the sex pheromone of the female moth to locate their host. Lewis (1982) first implicated volatile chemicals emanating from the abdominal tips of virgin female moths of *Heliothis zea* (Boddie) as a kairomone for *Trichogramma* spp. A synthetic sex pheromone blend of *H. zea* increased rates of parasitization of *H. zea* eggs by *Trichogramma*

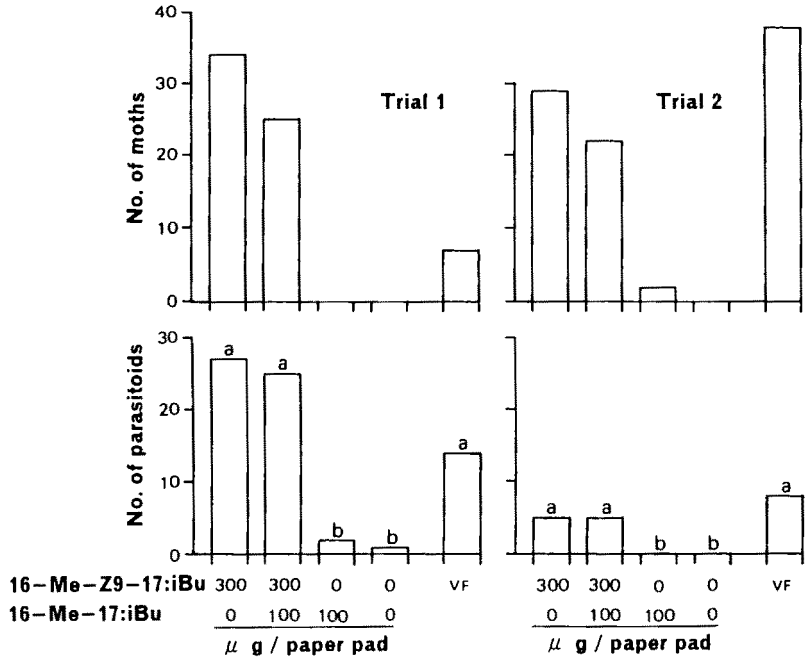

FIG. 1. Total trap catches of *T. euproctidis* (bottom) and male *E. taiwana* (top) with synthetic sex pheromone components, 16Me-Z9-17:iBu, 16Me-17:iBu, or a blend of 16Me-Z9-17:iBu and 16Me-17:iBu (1995, Okinawa). Data were transformed to $\sqrt{(x + 0.5)}$ and submitted to analysis of variance. Means followed by the same letter are not significantly different at the 5% level by Tukey's test. VF: three virgin female moths (1-3 days old).

spp. in greenhouse and field experiments. A similar finding was reported for the egg parasitoid, *Telenomus remus* Nixon; females showed increased rates of parasitization in the presence of volatile substances from the abdominal tips of *Spodoptera frugiperda* (J. E. Smith) or two compounds of the sex pheromone of this moth (Nordlund et al., 1983). In both these cases, however, the calling site of the female moth is not necessarily its oviposition site. A response, however, by these egg parasitoids to the host pheromone likely increases the possibility of their locating habitats in which the female moths have recently oviposited or are actively ovipositing eggs. In phoretic egg parasitoids, the link between parasitoid response to the adult pheromone and finding of the host eggs seems to be much tighter than in nonphoretic egg species.

Virgin female *E. taiwana* exposed in the field attracted significantly more female *T. euproctidis* than mated ones (Table 1). This suggests that the egg

parasitoids were attracted to the sex pheromone emitted by virgin female moths. Nevertheless, a small number of parasitoids was also attracted to mated moths. This may be due to sex pheromone adsorbed on the abdominal scale hairs as she calls. Because the sex pheromone is a high-molecular-weight, but volatile, pheromone, it may continue to attract egg parasitoids after the female moth has mated.

Two compounds were isolated and identified from abdominal tips of female *E. taiwana*: 16Me-Z9-17 : iBu, and 16Me-17 : iBu (Yasuda et al., 1995). Many *T. euproctidis* were attracted to 16Me-Z9-17 : iBu alone or a blend of 16Me-Z9 : iBu and 16Me-17 : iBu, but they were not attracted to 16Me-17 : iBu alone. These results showed that 16Me-Z9-17 : iBu is essential to attract the phoretic egg parasitoid. Male moths were attracted also by 300 μg of 16Me-Z9-17 : iBu alone or a blend of 300 μg of 16Me-Z9-17 : iBu and 100 μg of 16Me-17 : iBu, but no males were caught with 100 μg of 16Me-17 : iBu alone.

More parasitoids were caught in trial 1 than in trial 2. The traps in trial 1 may have attracted more individuals than those in trial 2 because trapping occurred in trial 1 over a longer period of time.

These experiments support the hypothesis that the phoretic egg parasitoid, *T. euproctidis*, uses the sex pheromone of the moth, *E. taiwana*, as a kairomone to locate the female moth, and through her the host eggs.

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BEHAVIORAL RESPONSES OF *Spodoptera littoralis* MALES TO SEX PHEROMONE COMPONENTS AND VIRGIN FEMALES IN WIND TUNNEL[1]

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Abstract—The major component of the sex pheromone of female *Spodoptera littoralis*, (*Z*,*E*)-9,11-tetradecadienyl acetate (**1**), elicited all steps of the male behavioral sequence, i.e., wing fanning and taking flight, oriented upwind flight and arrival to the middle of the tunnel, close approach and contact with the source. The activity was equivalent to that elicited by virgin females. In the range of doses tested, the dosage of **1** had no significant effect on the number of source contacts. Male response was significantly affected by light intensity, being optimum at 3 lux. Activity of the minor components (*Z*)-9-tetradecenyl acetate (**2**), (*E*)-11-tetradecenyl acetate (**3**), tetradecyl acetate (**4**), (*Z*)-11-tetradecenyl acetate (**5**), and (*Z*,*E*)-9,12-tetradecadienyl acetate (**6**) was significantly lower than that of the major component when assayed individually. In multicomponent blends compound **4** appeared to strongly decrease the number of males arrested at the source, the effect being particularly important when compound **5** was present in the blend. Results of single sensillum experiments confirmed the existence of two main physiologically distinct sensillar types. The most common type of sensilla contained a neuron that responded specifically to compound **1**. A second type of sensilla, located laterally on the ventral sensory surface, contained two receptor neurons responding to compound **6** and to (*Z*)-9-tetradecenol. Among short sensilla, one hair responded to compound **4** and could represent a minor sensillar type. No sensory neuron was found to detect the other minor pheromone compounds **2**, **3**, and **5**.

Key Words—Wind tunnel, behavior, single sensillum recording, *Spodoptera littoralis*, Lepidoptera, Noctuidae, Egyptian armyworm.

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[1]Dedicated to the memory of the late Prof. Félix Serratosa (1925–1995).

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INTRODUCTION

Wind tunnels have been widely used as a reliable technique to study the orientation mechanisms of flying insects to their sex pheromones (Miller and Roelofs, 1978; Cardé, 1984; Mafra-Neto and Cardé, 1994), as well as for evaluation of the mating disruption process (Sanders, 1982) and inhibition induced by specific synthetic compounds (Preiss and Priesner, 1988).

The sex pheromone of the Egyptian armyworm *Spodoptera littoralis*, an important pest of cotton and vegetable crops in Europe, Asia, and Africa, was first identified by Nesbitt et al. (1973) as a mixture of (*Z*,*E*)-9,11-tetradecadienyl acetate (**1**), (*Z*)-9-tetradecenyl acetate (**2**), (*E*)-11-tetradecenyl acetate (**3**), and tetradecyl acetate (**4**) (Figure 1). The pheromone composition, however, varies with the origin of the strain analyzed. Thus, while Dunkelblum et al. (1982) in Israel reported that the sex pheromone complex was a mixture of **1** (33%), **2** (46%), **3** (9%), (*Z*)-11-tetradecenyl acetate (**5**) (7%), (*Z*,*Z*)-9,11-tetradecadienyl acetate (4%), and (*Z*,*E*)-9,12-tetradecadienyl acetate (**6**) (0.5–1%), Tamaki and Yushima (1974) found that abdominal tip extracts from Kenyan insects contained a mixture of only **1** and **6**. Different compositions were also found by Campion et al. (1980) from calling virgin females of Crete, Israel, and Egypt. Thus, whereas volatiles of virgin females from Egypt contained much larger amounts of compounds **4** and **2** + **3** than **1**, the Israeli strain showed compounds **4** and **5** as the major components, and the Cretan stock produced only saturated acetate **4**. The small number of insects available, however, made the results of this study of limited value. In previous studies carried out on our laboratory strain, Martínez et al. (1990) found that the pheromone gland contained a mixture of compounds **1–5** in the ratio 66:12:11:2:9, respectively. The estimated amounts

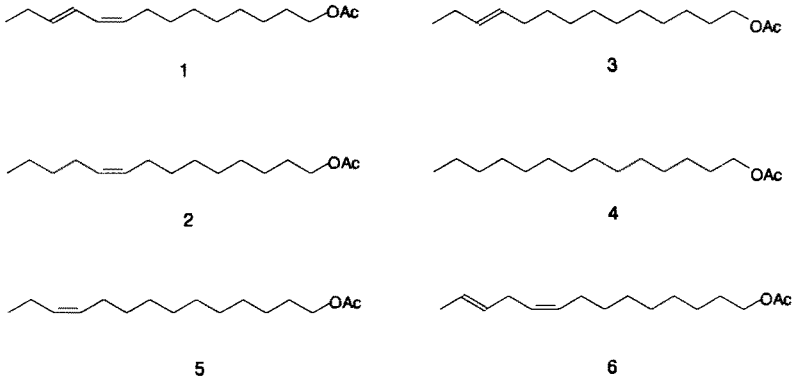


FIG. 1. Structures of compounds **1–6** tested.

of these compounds per female were ca. 40, 7, 7, 1, and 6 ng, respectively. The difference in composition of the pheromone of our strain relative to others led us to study the behavioral activity induced by each component found in the gland alone and in blends with the major compound **1.**

Although field tests are the definitive experiments to assess pheromonal activity (Ridgway et al., 1990), behavioral assays to determine male response to pheromone components in comparison with virgin females are also beneficial for gaining insight into the communication system of the species (Baker and Cardé, 1984; Löfstedt et al., 1985; Linn et al., 1987). In this regard, only one report describing minor component effects on male behavior of Egyptian and Cretan origins has been found in the literature (Haines, 1983). In this paper we report behavioral activity of components **1–5** found in the female gland of our strain, alone and in blends with the major compound **1.** Activity of diene **6,** absent in our population but present in others (see above), and comparison of the activity of the major component and the natural blend with that of virgin females is also presented. Single sensillum experiments (Kaissling, 1979) were also conducted in order to establish the existence of specific receptor cells for the pheromone components found in the gland.

METHODS AND MATERIALS

Insects. Insects were obtained from a laboratory colony taken from the Station de Zoologie (INRA, Montfavet, France) and reared in our laboratory on a slightly modified artificial diet from the one previously reported (Poitout et al., 1972). Modifications include replacement of aureomycin chlorohydrate by Wesson salt mixture (1%, Sigma) and addition of 0.08% of 35% aq. formaldehyde. Pupae were sexed, placed in groups of 20–25 in 20- × 20-cm plastic boxes, and maintained in a climatic chamber on a 16L:8D regime at 25 ± 1°C with 60–70% relative humidity until emergence. Adults were separated daily by age and provided with 10% sucrose solution.

Chemicals. Compounds **1–3** were obtained from Sigma Chem. Co. (St. Louis, Missouri) and their purity (>95%) was checked by GLC on a SPB-5 fused silica capillary column. Stereochemical purity of **1** was *Z,E*:*E,E* 96:4. Compounds **4** and **5** were prepared by acetylation of tetradecyl alcohol and (*Z*)-11-tetradecenol (Sigma), respectively, with acetyl chloride 99%+ (Aldrich Chemie, Steinheim, Germany) followed by purification by column chromatography to assess a >98% purity by GLC analysis. Just prior to the experiments, solutions were prepared by dissolving the compounds in 100 μl of nanograde hexane. The required volume to achieve the test doses (1–1000 μg) was applied to a 1.5- × 1-cm cotton wick and the solvent evaporated. The wicks were discarded after use.

Wind Tunnel. The tests were performed in a glass wind tunnel 180 cm long, 55 cm wide, and 50 cm high. The frame of the tunnel was made of aluminum and access was gained by two sliding doors, each one covering one half of the tunnel. The air was pushed through by a centrifugal fan and pulled out of the building with the aid of an exhaust blower. The fan and blower worked simultaneously and their action was carefully regulated by a potentiometer to avoid undesired turbulence in the moving air. Turbulence was controlled through a SO_3-containing airflow tester (Drägerwerk Aktiengesellschaft, Germany). The airstream was purified through a 2-cm-thick glass wool bed and conducted through two consecutive nylon screens to smooth the airflow and get a laminar regime through the tunnel. A 58-W red fluorescent light located 16 cm above the tunnel provided the required illumination. Light intensity was adjusted to 3–10 lux by covering the top of the tunnel with red cellophane paper strips. A white board containing irregular-shaped, dark brown spots was placed on the floor of the tunnel since striped patterns may induce unexpected effects on the orientation of flying insects (David, 1982). The airspeed was 45–55 cm/sec, the temperature of the experiment room was 22 $\pm$ 1°C, and the relative humidity 60 $\pm$ 5%. The wind tunnel was thoroughly cleansed with acetone before every change of formulation.

Bioassays. Newly emerged moths were separated from the remaining pupae twice a day and placed into 31- $\times$ 12- $\times$ 21-cm plastic boxes. Only insects on their first and second scotophase were tested since they had shown maximum EAG responses in comparison to older insects (Murgó and Guerrero, unpublished data). At the onset of the scotophase, males were distributed in groups of five individuals, placed in Petri dishes, and maintained in the dark for 3 hr. They were then allowed to acclimate to the tunnel conditions for 60 min and utilized only once. The males were taken from their containers, placed over a filter paper on a stainless steel jack at 130 cm downwind from the source, and allowed 5 min to respond. When virgin females were used as the pheromone source, five individuals (10–35 hr old) were placed in 6.5- $\times$ 4 $\times$ 3-cm stainless steel cages of 0.2- $\times$ 0.2-cm mesh and suspended about 18 cm from the top and 40 cm from the upwind end of the tunnel. Males were introduced into the tunnel only after the virgin females had adopted the calling position. To study the effect of synthetic chemicals, cotton wicks containing the compounds were hung on the same holders. The experiments were carried out 3–6 hr into the scotophase since female calling behavior reaches its maximum 3–9 hr into the dark period (Dunkelblum et al., 1987). Responses of 15–20 males were recorded per day of experiment with a minimum of two replicates per formulation.

Males were scored according to the following behavior: wing fanning and taking flight, arrival at the middle of the tunnel (65 cm), close approach to the lure (ca. 10 cm), and contact with the source. In the tests with individual compounds, latency, i.e., delay in time spent by males to leave the filter paper,

was also considered. Only those males arrested at the source for a minimum period of 5 sec were recorded as source contacts. The effect of a specific formulation was calculated as the number of males showing a particular behavior in comparison with the total number of insects released. The results were analyzed for significance using a chi-square 2 × 2 test of independence (Sokal and Rohlf, 1969).

Electrophysiology. Single sensillum recordings (SSRs) were performed on whole insect preparations according to the standard tip recording technique (Kaissling and Thorson, 1980). Glass electrodes were filled with sensillar saline for the recording electrode and with hemolymph saline for the reference electrode. After cutting the more distal segments, the tip of the antenna was covered with the hemolymph saline. The tips of several sensilla were cut off using sharpened forceps, and the recording electrode was slipped over the cut end of one hair. The SSR responses were filtered (150–5000 Hz) and amplified (×1000). The recordings were stored on a PC-AT microcomputer via a DASH 16 analog-to-digital conversion board. Acquisition and analysis of the recordings were performed by programs we developed in Asyst (McMillan Software Co.). Records were plotted with Awave, a software developed by F. Marion-Poll in Visual C++.

In addition to pheromone components **1–6,** other pheromone analogs were also tested on several trichoid sensilla, i.e., dodecyl acetate, (*Z*)-7-dodecenyl acetate, (*Z*)-9-dodecenyl acetate, and (*Z*)-9-tetradecenol, in a randomized order. The olfactory stimulations were achieved by blowing a puff of air (0.5 liters/min) of 1 sec duration through a Pasteur pipet containing a filter paper, on which 0.1, 0.5, or 5 μg of the test compound diluted in 1 μl of hexane had been deposited. The antenna was continuously flushed with humidified air (1.0 liter/min, 100% relative humidity). Receptor neurons tuned to compound **4** were searched for in olfactory hairs sampled among all different morphological classes and locations of sensilla trichodea. In addition, the activity of binary mixtures **1** + **4** were compared to that displayed by the individual compounds alone in order to check for a possible interaction of these two components at the receptor cell level.

RESULTS

Male Response to Major Component 1. The effect of dosage of major component **1** on male behavioral response is shown in Figure 2. In the range of doses tested (1–1000 μg), dosage had no significant effect on the number of males displaying every stage of the courtship sequence. In contrast, light intensity exerted a clear effect on male responses to major component **1,** particularly at moderate dose (Figure 3). Thus, when 1, 3, and 10 lux were tested, males

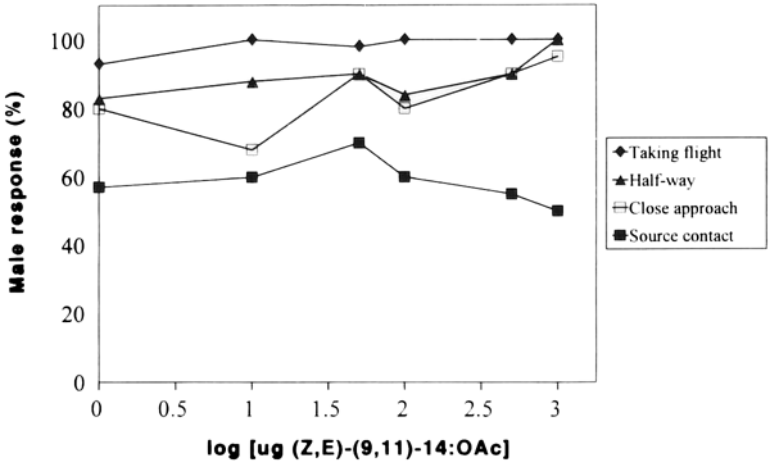


FIG. 2. Percentage of *Spodoptera littoralis* males exhibiting all types of behavior in response to various doses of (*Z*,*E*)-9,11-tetradecadienyl acetate (**1**) in wind tunnel at an airspeed of 45 cm/sec and 3 lux of light intensity.

displayed highest responses at 3 lux and significantly higher responses at 50- to 100-μg doses than those elicited with other illuminations. The effect was dose-dependent, the difference in number of males contacting the source under the three light intensities was higher at higher concentrations.

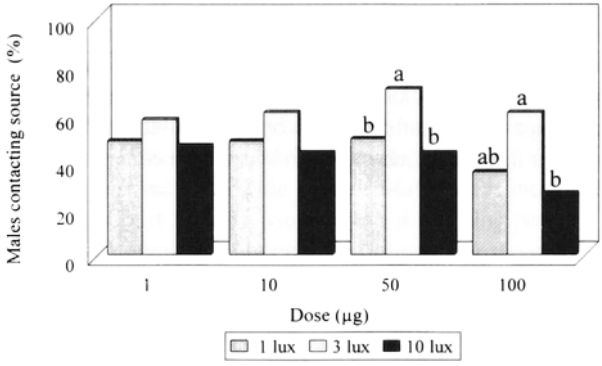


FIG. 3. Percentage of *Spodoptera littoralis* males contacting the source in response to several doses of (*Z*,*E*)-9,11-tetradecadienyl acetate (**1**) under different light intensities. Bars topped by different letters within a dose are significantly different at $P < 0.05$ (chi-square 2 × 2 test).

Male Response to the Minor Components 2–6. The minor components **2–5,** found in our strain, as well as diene **6** found in some strains, were assayed individually at several concentrations (100–1000 μg). Little activity was generally elicited at any of the doses tested, the activity being significantly lower than that displayed by the major component **1** ($P < 0.05$) (Figure 4). Lack of response was evident at the earliest stages of behavior, i.e., wing fanning and higher latency (> 120 sec) in comparison with insects responding to major component **1** (60–100 sec). In most cases the number of source contacts was negligible, so activity was evaluated in terms of number of males performing close approach. At the 100-μg dosage, compound **2** was the most active minor component ($P < 0.05$). It was able to induce landing of 35% of males at that concentration. Compound **3** was also active, the effect being higher at increasing concentrations, although activity was not significant relative to the other minor components. Compounds **4** and **5,** in turn, were only able to elicit close approach to the source in 20–30% of males, whereas compound **6** showed moderate attractivity only at 1000 μg.

Male Response to Binary Mixtures. The effects induced by binary mixtures of **1** with minor components **2–6** in a similar ratio to that found in the pheromone gland were also studied. Cotton wicks were loaded with previously prepared

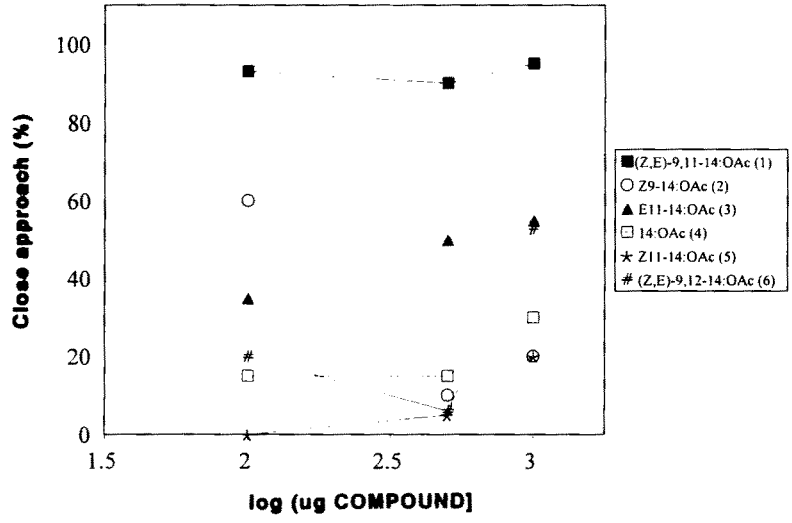


FIG. 4. Percentage of *Spodoptera littoralis* males performing close approach behavior in response to various doses of Z9–14:OAc (**2**), *E*11–14:OAc (**3**), 14:OAc (**4**), Z11–14:OAc (**5**), and (*Z,E*)-9,12–14:OAc (**6**) in wind tunnel. Responses to (*Z,E*)-9,11-tetradecadienyl acetate (**1**) are given for comparison.

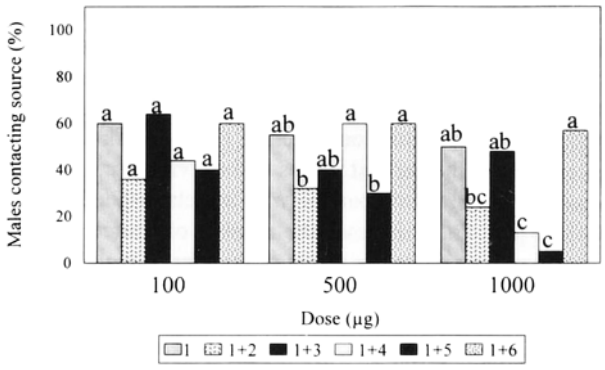


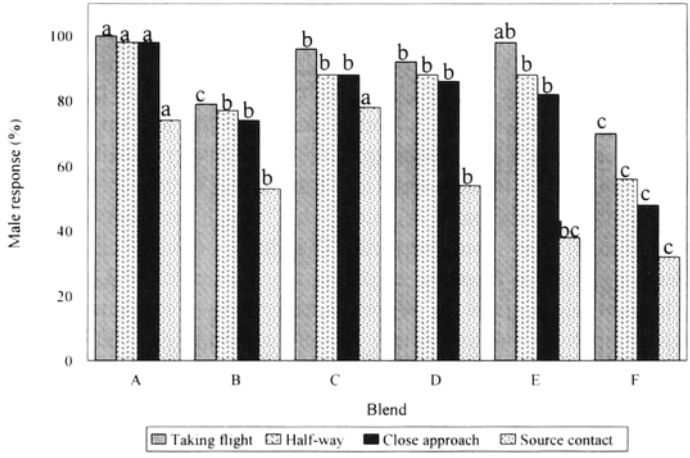
FIG. 5. Percentage of *Spodoptera littoralis* males contacting the source in response to binary mixtures of (*Z*,*E*)-9,11-tetradecadienyl acetate (**1**) with *Z*9-14:OAc (**2**), *E*11-14:OAc (**3**), 14:OAc (**4**), *Z*11-14:OAc (**5**), and (*Z*,*E*)-9,12-14:OAc (**6**) at different doses. Bars bearing different letters within a dose are significantly different at $P < 0.05$ (chi-square 2 × 2 test).

mixtures of 100, 500, and 1000 μg of **1** and the corresponding amount of the minor compounds (Figure 5).

In general, these binary blends did not increase the number of source contacts elicited by compound **1.** Differences in activity between the major component and mixtures increased with the concentration, being significant ($P < 0.05$) at the highest doses (500–1000 μg). Among the binary mixtures, formulation **1** + **3** was the most active, but the number of source contacts was very similar to that displayed by compound **1** alone. A slightly lower effect was elicited by mixture **1** + **2,** with activity being independent of the concentration assayed. The effect of the dosage was particularly evident when blends **1** + **4** and **1** + **5** were tested at 1000 μg, wherein both mixtures elicited a notable inhibition of landing response. The effect was significant ($P < 0.05$) relative to the one displayed by the major compound alone. Compound **6** was completely ineffective when mixed with **1** at every dosage tested, in comparison with the activity induced by the major component alone.

Male Response to Multicomponent Blends. In order to compare the response to synthetic pheromone blends with that elicited by virgin females, several multiple combinations of compounds **1–5** were assayed in similar ratios to the one found in the gland. Six blends, A–F, were prepared by adding the corresponding amounts of components **2–5** to 1000 μg of the major component **1.** The final formulations were the following: A: **1** + **2** + **3,** 75:13:12; B: **1** + **2** + **3** + **4,** 66:12:11:11; C: **1** + **2** + **3** + **5,** 67:12:11:9; D: **1** + **2** + **3** + **4** + **5,** 67:12:11:0.5:9; E: **1** + **2** + **3** + **4** + **5,** 66:12:11:2:9; F: **1** + **2** + **3** + **4** + **5,** 61:8:10:10:11.

Formulation E, which mimicked the natural pheromone blend, was, surprisingly, one of the less effective blends, with only 38% of males landing at the source (Figure 6). Formulation A, in contrast, was the most active, with 74% of males contacting the lure. Blend C was also very effective in eliciting complete flights to the source, and although the number of males taking flight and approaching the source was lower than with blend A ($P < 0.05$), there was no significant difference in the number of source contacts. Activity of formulation C was not significantly higher than that of virgin females, but induced a significantly higher number of male arrests at the source than compound **1** alone ($P < 0.05$, Figure 7). Addition of **4** to blend A (formulation B) significantly decreased male response. This effect was confirmed when the same compound was added to formulation C to make blend E. In this case, however, only the number of contacts with the source was significantly diminished. The same effect was noticed with formulation D, containing only 0.5% of compound **4.** The difference in behavior elicited relative to blend C was also significant ($P < 0.05$). Furthermore, formulation B, containing 11% of compound **4,** significantly reduced the number of males displaying all types of behavior in comparison with blend A. The inhibitory effect induced by **4** can be also noticed in



A: 1 + 2 + 3 (75:13:12)
B: 1 + 2 + 3 + 4 (66:12:11:11)
C: 1 + 2 + 3 + 5 (67:12:11:9)
D: 1 + 2 + 3 + 4 + 5 (67:12:11:0.5:9)
E: 1 + 2 + 3 + 4 + 5 (66:12:11:2:9)
F: 1 + 2 + 3 + 4 + 5 (61:8:10:10:11)

FIG. 6. Percentage of *Spodoptera littoralis* males exhibiting all types of behavior in response to multicomponent mixtures of (*Z*,*E*)-9,11-tetradecadienyl acetate (**1**) with *Z*9-14:OAc (**2**), *E*11-14:OAc (**3**), 14:OAc (**4**), and *Z*11-14:OAc (**5**). Values corresponding to the same type of behavior have been compared and bars topped by different letters are significantly different at $P < 0.05$ (chi-square 2 × 2 test).

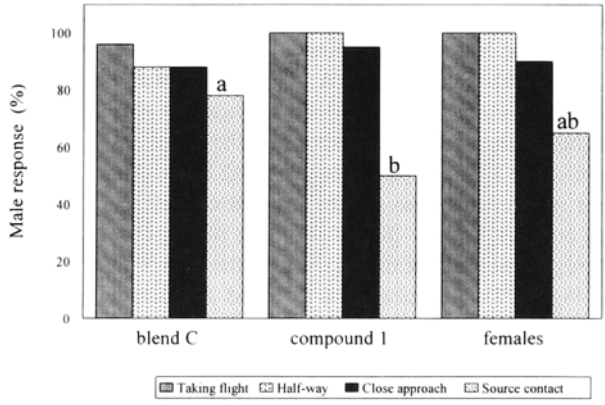


FIG. 7. Comparison of male responses to blend C, major compound **1**, and virgin females. Bars topped by different letters are significantly different at $P < 0.05$ (chi-square 2 × 2 test).

formulation F, wherein the number of males performing all behaviors was also significantly lower ($P < 0.05$) in comparison to those responding to blend C (Figure 6).

Single Sensillum Recordings. The activity of more than 100 long and 100 short sensilla trichodea chosen at random in the proximal half of the antenna was recorded. When the amplitude of their action potentials could be clearly distinguished from each other, A and B designate the sensory neurons firing action potentials of high and low amplitude, respectively. Most short and long trichoid sensilla housed a large-spike cell responding only to compound **1** (Figure 8a). In some of these hairs, a second cell, which fired action potentials of smaller amplitude, was also observed. The firing activity, however, did not significantly increase in response to pheromone compounds. Long sensilla trichodea, located laterally on the ventral sensory side of the antenna, housed A cells responding to compound **6** and B cells responding to (*Z*)-9-tetradecenol (Figure 8b,c). No response to **4,** even at 5 μg, was recorded from these two sensillar types. However, one short sensillum trichodeum showed a different cell response pattern. Its firing activity consisted of a single class of action potential amplitude. This sensillum neither responded to compounds **1, 6,** and (*Z*)-9-tetradecenol at 500 ng and 5 μg doses nor to compound **4** (500 ng), but stimulations with 5 μg of the latter component elicited significant responses (Figure 8d). Despite extensive efforts, no other hair displaying the same type of response could be found. Numerous short sensilla did not respond to any of the pheromone molecules assayed.

Sixteen sensilla from three males containing a receptor neuron tuned to

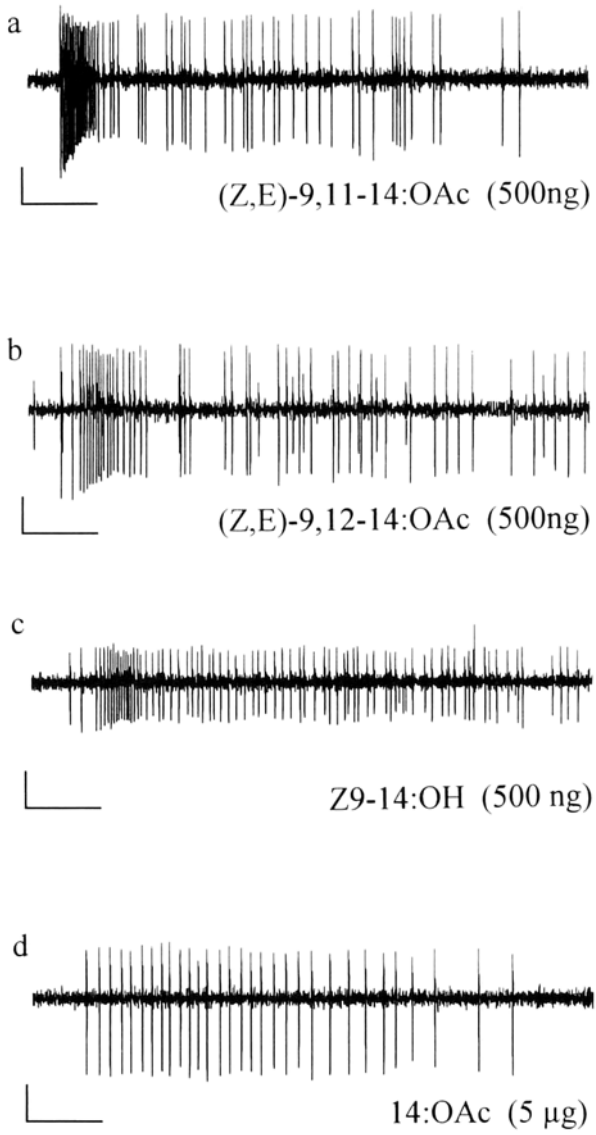


FIG. 8. Single sensillum recordings from the three types of sensilla trichodea: (a) from a sensillum responding to (*Z,E*)-9,11-tetradecadienyl acetate (**1**); (b) and (c) from a sensillum responding to (*Z,E*)-9,11-tetradecadienyl acetate (**1**) (A cell), and to *Z*9–14:OH (B cell); and (d) from a sensillum responding to 14:OAc (**4**). Scale bars: 200 msec, 0.5 mV. Stimulation duration: 1 sec.

compound **1** were subjected to compounds **1** (100 ng), **4** (10, 100 ng), and mixtures of **1** + **4** (100 + 10 ng, 100 + 100 ng). Compound **4** alone was inactive but when mixed with compound **1**, it slightly increased the firing frequency by 2–5 spikes/sec, but the difference was not significant (data not shown).

DISCUSSION

Our results show that in our strain, major compound **1**, whose presence in the gland accounts, on average, for 66% of the total amount of pheromone components, is able to produce alone all steps of the behavioral sequence in males. The activity is similar to that displayed by virgin females. The response level is clearly dependent on light intensity, particularly at 50 μg dosage and higher. Wind velocity had practically no significant effect at speeds ranging from 45 to 80 cm/sec, whereas at 40 cm/sec only 25% of males initiated upwind flight towards the source (data not shown). This is in agreement with the experiments of Haines (1983) on other strains.

Low loadings (1 μg) of the major component are sufficient to promote all behaviors of the mate-finding sequence, but higher doses do not impair the ability of males to orient. Many insects respond to low amounts of chemicals in their flight orientation behavior, i.e., *Mamestra suasa* (Frérot et al., 1989), *Grapholita molesta* (Linn and Roelofs, 1983; Linn et al., 1988), *Agrotis segetum* (Löfstedt et al., 1985), *Laspeyresia pomonella* (Preiss and Priesner, 1988), *Lymantria dispar* (Charlton et al., 1993), or *S. litura* (Kawasaki, 1986), among others. The high attractivity of **1** had been previously noticed in the field by Kehat et al. (1976), who found that 1 mg of the chemical was equivalent in effectiveness to a trap baited with virgin females.

In general, little activity was displayed by the minor components when assayed individually, the lack of response being evident at the earliest steps of the behavioral sequence. However, compounds **2** and **3** evoked source contacts in 20–30% of males at 100 μg and 500 or 1000 μg, respectively (data not shown). Compound **2** was found to be encoded by a specific receptor by Priesner (1979), but we did not find a specific receptor cell for **2.** In the field, compounds **2** and **5** behaved as inhibitors of the male activation response induced by compound **1** (Campion et al., 1980), although Kehat et al. (1976) found that addition of small amounts of **5** to the "pure" major component **1** in the Israeli strain markedly enhanced the activity of the diene. Acetate **6,** whose presence in other strains appeared essential for a maximum trap catch in the field (Kehat et al., 1976; Dunkelblum et al., 1982), elicited activation and orientation to the plume only at high doses but failed to induce close approach to the source.

Binary mixtures of the minor components with major compound **1** did not significantly increase the number of males responding in comparison with com-

pound **1** alone. There was, however, a significant inhibitory effect in the number of contacts elicited by compounds **4** and **5** at high dosage (1000 μg). The effect induced by **4** is in agreement with the results of Haines (1983) on a Cretan population, wherein addition of ≥ 10% of **4** significantly reduced the percentage of males responding. The same mixture, however, did not elicit any appreciable effect on the Egyptian strain. These results suggest that minor components **4** and **5** exert their arrestment activity at a close distance to the source and at relatively high atmospheric concentrations, in a similar way to the activity shown by dodecyl acetate in *Trichoplusia ni* (Linn and Gaston, 1981).

The effect exerted by compound **4** was confirmed when multiple component blends B–F were assayed. Comparison of formulations D and E with C clearly shows an inhibition effect in the number of males arrested at the source, the presence of only 0.5% of **4** being enough to induce a significant decrease of source contacts. The effect was dose-dependent, the highest inhibition of response being elicited by composition F containing 10% of the minor component. The effect was specially remarkable when compound **5** was also present in the pheromone composition (compare formulations B and F). Compound **4** appears, therefore, to be a close-range inhibitor, but depending on the presence and concentration of **5**, it may also inhibit other behaviors, such as wing fanning, orientation to the plume, and close approach to the source.

The most active formulations were A and C, the latter inducing slightly lower activation and orientation to the plume but with similar numbers of males arrested at the source. Compound **5**, present in formulation C, appears, therefore, to be a behaviorally neutral minor component. The high activity elicited by blends A and C, analogous to that of virgin females, and the inhibition shown by compound **4** suggest that perhaps females do not release this minor component. Despite extensive effort, we have been unable to detect saturated acetate **4** in aeration experiments of female volatiles.

The presence of compound **4** in the pheromone gland induced us to look for a specific receptor cell and to study its peripheral detection by male antennae. Single sensillum recordings confirmed the existence of two main physiological types of sensilla trichodea on the male antenna, as previously described by Ljungberg et al. (1993). Thus, despite differences in the composition of their sex pheromones, the two populations show the same sensillar organization. In addition, among short hairs, one different sensillum responded to saturated acetate **4**. No sensillum of the same type was, however, recorded in further experiments, which can be interpreted in two ways. Either hairs of this type are present in very low numbers on the antenna, as is the case for the (*Z*)-9-tetradecenyl acetate receptor cell in *Agrotis segetum* (Löfstedt et al., 1982), or the hairs responding to the inhibitor may have been undersampled due to their location or size. Considering the very weak EAG activity of compound **4** (Nes-

bitt et al., 1973; Ljungberg et al., 1993), the former possibility seems more likely. Responses from this sensillum were recorded only to 5 μg of compound **4**, ten times higher than the dose required for eliciting activity on the other two sensillar types. This high threshold may be due to the absence of tuning of this sensillum to the minor component, or it may originate from the experimental conditions, which include cutting the tip of the sensilla and covering its distal part. On a short hair, this may result in a considerable diminution of the sensory zone in contact with the stimulating pheromone molecules.

In summary, the major component **1** is a very effective attractant for the Spanish strain of *Spodoptera littoralis* males in behavioral studies, its activity being equivalent to that elicited by virgin females. Among the minor components present in the gland and in multicomponent blends, compound **5** is behaviorally neutral, while **4** appears to be a close-range inhibitor of arrestant behavior.

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SEX PHEROMONE OF *Stenoma cecropia* MEYRICK (LEPIDOPTERA: ELACHISTIDAE)

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Abstract—(*Z,E*)-9,11-tetradecadienal (*Z*9,*E*11-14:Ald; 11%), (*Z,E*)-9,11,13-tetradecatrienal (*Z*9,*E*11, 13-14:Ald; 67%), (*Z,E*)-9,11-tetradecadienyl acetate (*Z*9,*E*11-14:Ac, 5.5%), and (*Z,E*)-9,11,13-tetradecatrienyl acetate (*Z*9,*E*11,13-14:Ac; 16.5%) were identified in the extracts of female pheromone glands of *Stenoma cecropia* (Lepidoptera: Elachistidae). From electroantennograms and single sensillum recordings, receptors to *Z*9,*E*11,13-14:Ald and *Z*9,*E*11-14:Ald were found on male antenna. Behavioral data were obtained from olfactometric tests in the laboratory and field trapping experiments in Colombia. It appeared that a blend of *Z*9,*E*11,13-14:Ald (83%) and *Z*9,*E*11-14:Ald (17%) was attractive to males. These aldehydes are assumed to be components of the sex pheromone of *S. cecropia*, whereas the acetates found in gland extracts might be precursors of the pheromone.

Key Words—Lepidoptera, Elachistidae, *Stenoma cecropia*, sex pheromone, identification, electroantennography, single sensillum recording, field trapping (*Z,E*)-9,11-tetradecadienal, (*Z,E*)-9,11,13-tetradecatrienal.

INTRODUCTION

The moth *Stenoma cecropia* Meyrick belongs to a neotropical subfamily (Stenominae) of the Gelechioidea. These moths were considered as Oecophoridae by

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Hodges (1974). More recently, Minet revisited the taxonomic status of Stenominae and included them in the family Elachistidae on the basis of the abdominal articulation of the nymph (Minet, 1989). The adults of *S. cecropia* look like colorful tortricids, but the larvae live in fixed bags and feed on several forest trees, including the leaflets of Palmaceae. In oil palm plantations of central Colombia, the populations of this insect can reach huge levels, and the threshold for insecticidal treatment is 80 larvae per leaf (Genty, 1978). Severe defoliations by this insect may decrease the yield of oil palm trees by as much as 15–20%. We undertook the identification of the sex pheromone of *S. cecropia* to provide a useful tool for monitoring the populations of the moth.

The chemical aspects relating to the synthesis of pheromone components have been partly published (Ramiandrasoa and Tellier, 1990; Tellier and Descoins, 1990). We present here the full identification study together with biological data.

METHODS AND MATERIALS

Insects

Preliminary trials to send live insects to France were very unsatisfactory, because 90% of the larvae or pupae died. It was thus decided in 1987 to extract the pheromone in central Colombia by using a field laboratory in a 9000-ha plantation (San Alberto, Cesar). The pupae were collected daily in their bags and sexed in the laboratory. Half the pupae and emerging adults were then kept under natural conditions (temperature varying between 28 and 40°C, 100% relative humidity, and daylight from 06:30 to 18:30 hr). The other half of the insects were kept in a room under similar conditions except that light was provided from 04:30 to 16:30 hr.

The insects used for olfactometric tests, electroantennographic (EAG) recordings, and single-sensillum recordings were collected as young pupae in the field and brought back to France courtesy of traveling colleagues. They were sexed and kept at 27°C with 75% relative humidity under a 12–12 hr photoperiod until emergence of the adults. The male moths used in these laboratory studies were 1 or 2 days old.

Pheromone Extraction

As shown by observation of traps baited with virgin females, the period of sexual activity of *S. cecropia* begins half an hour before dusk and lasts 2 hrs. In San Alberto, the pheromone was extracted between 18:30 and 20:30 hr (insects under natural photoperiod) or between 16:30 and 18:30 hr (insects under shifted light regime).

The last three abdominal segments of 1 or 2-day old virgin females were clipped and soaked for 12 hr in 1 ml hexane (Carlo Erba Codex Depurato) at −5°C. The extract then was filtered through glass wool, reduced to ca. 100 μl under a N_2 flow and kept at −30°C until used. Three extracts of, respectively, 684, 759, and 557 female abdominal tips were made using this method from February 27 to March 18, 1987.

Pheromone Analysis

The sex pheromone extracts were analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS). GC analyses were performed on a Hewlett-Packard 5890 chromatograph with flame ionization detector (FID) and a Ross injector heated at 240°C, equipped either with a polar column (30-m WCOT CPSil-88 CB, 0.22 mm ID; Chrompack, Middleburg, The Netherlands) operated isothermally at 180°C, or with a nonpolar column (25-m WCOT CPSil-5 CB, 0.22 mm ID; Chrompack) heated from 160 to 220°C at 5°C/min. GC-MS was performed on a Girdel 32 gas chromatograph linked to a Nermag R 10-10C quadrupole mass spectrometer controlled by a SIDAR data acquisition system. The chromatograph was fitted with a fused silica capillary column (25-m WCOT CPSil-5 CB, 0.22 mm ID; Chrompack) and operated isothermally at 180°C. Mass spectra were obtained by electron impact (EI) at 70 eV scanning from 40 to 350 amu, and by chemical ionization (CI) at 92.5 eV with ammonia (NH_3) and isobutane (i-C_4H_{10}) as the reactant gas. Pheromone compounds were identified by comparing their GC retention times and mass spectra with those of chemicals synthesized in the laboratory (see below).

Synthesis of Chemicals

We report herein the synthesis of (*Z*,*E*)-9,11,13-tetradecatrien-1-al. For some years, several laboratories have been interested in synthesis of 1,3,5-undecatrienes (Näf et al., 1975; Ratovelomanana and Linstrumelle, 1987; Andreini et al., 1987), which are reported to have odors highly appreciated in perfumery. The need for regio- and stereochemically pure compounds for biological tests is well recognized. A number of stereoselective methods (Ratovelomanana and Linstrumelle, 1987; Andreini et al., 1987) for obtaining these nonfunctionalized trienes have been described. In our case the approach that has given the best results was based upon the method of Rossi (Andreini et al., 1987), which involved two sequential palladium-catalyzed cross-coupling reactions. A first coupling between a molar excess of commercial *E*/*Z* 1,2-dibromoethylene and adequate alkynylzinc bromides led to (*E*)-1-bromo-1-en-3-yne derivatives with a high stereoisomeric purity, since (*E*)-1,2-dibromoethylene reacts preferentially when it is blended with the *Z* stereoisomer. These intermediate bromoenynes were then coupled with vinylzinc bromide to afford the

corresponding 1,(*E*)-3-dien-5-ynes. The latter were allowed to undergo a *Z* stereoselective reduction to give 1,(*E*)-3,(*Z*)-5-trienes. The actual steps for synthesizing the desired trienal are shown in Figure 1. ^1H and ^{13}C NMR spectra were recorded on a Bruker AC 200 spectrometer [$CDCl_3$; δ(ppm) from TMS, *J* (Hz)]. Infrared spectra were measured on a Perkin-Elmer 397 spectrometer [neat, γ cm^{-1})]. Stereoisomeric purity of all products was determined by gas chromatographic analyses performed on a model 2900 Carlo Erba instrument equipped with silica capillary polar column (25-m WCOT FFAP 0.32 ID, H_2 carrier gas flow 25 ml/min, 1.2 b).

(E)-1-Terbutoxy-12-bromo-9-dodecyn-11-ene. To a solution of 1-terbutoxy-9-decyne (Tellier and Descoins, 1990) [10.5 g (0.05 mol) in 25 ml Et_2O and 25 ml THF] at 0°C were added 31 ml (0.05 mol) of 1.6 M *n*-BuLi in hexane. After 30 min at 20°C, alkynyl lithium was obtained. To this reagent was added at 0°C an anhydrous $ZnBr_2$ solution [12.4 g (0.055 mol)/50 ml THF]. The stirred mixture was warmed to 20°C for 30 min; then at 0°C were added successively solutions of $Pd(PPh_3)_4$ (3%) [1.7 g (1.5 mmol)/20 ml THF] and 46.5 g (0.25 mol) of *E/Z* (40/60) 1,2-dibromoethylene. The reaction mixture was allowed to warm to room temperature for 16 hr and then hydrolyzed by H_2SO_4 solution (1 N). After extraction with ether and evaporation of the solvent, the crude mixture was passed through a small silica gel column in order to remove the palladium catalyst (eluting with cyclohexane). The solvents were evaporated and the residue distilled to afford 7.9 g of bromoynene (50% yield); *E* purity ≥ 99%; bp, 120°C/0.1 Torr; *m/z*: 299, 301 (M-15); IR: 2250, 1200; ^1H NMR: 1.18 (s, 9H), 1.33 (m, 12H), 2.22 (m, 2H), 3.32 (t, 2H), 6.13 (dt, H^{11}), 6.56 (d, H^{12}) $J_{H^{11}/H^{12}}$ = 14, $J_{H^8/H^{11}}$ = 2; ^{13}C NMR: 19.5, 61.6, 72.3, 77.3, 93.2, 116.8, 118.1. Anal. calcd. for $C_{16}H_{27}BrO$: C, 60.95; H, 8.63. Found: C, 61.20; H, 8.77.

(E)-1-Acetoxy-12-bromo-9-dodecyn-11-ene. Ac_2O, 4.7 ml (0.05 mol), and 0.4 g (2.5 mmol) of $FeCl_3$ were successively added to a solution of 7.9 g (0.025

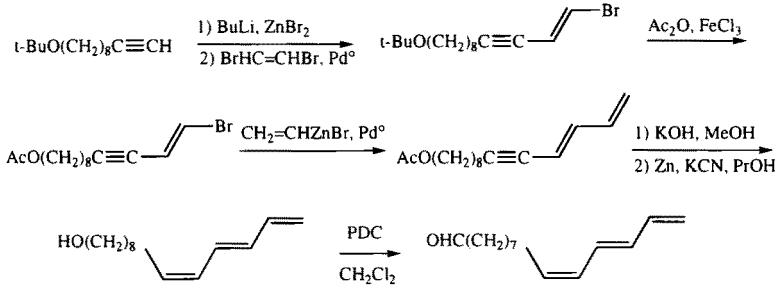

FIG. 1. Synthesis of (*Z,E*)-9,11,13-tetradecatrien-1-al.

mol) *t*-butyl ether in Et_2O (50 ml). The stirred solution was left at room temperature overnight. A saturated aqueous solution of Na_2HPO_4 (50 ml) was added and the mixture was stirred for 1 hr. The solid precipitate was removed by filtration. After extraction with ether and evaporation of the solvent, 6.9 g of acetoxybromoynene were obtained (92% crude yield); *E* purity (99%); *m/z*: 221 (M-80); IR: 2250, 1740; ^{1}H NMR: 1.2–1.7 (m, 12H), 2.03 (s, 3H), 2.25 (m, 2H), 4.04 (t, 2H), 6.16 (dt, H^{11}), 6.57 (d, H^{12}) $J_{H^{11}H^{12}}$ = 14, $J_{H^8/H^{11}}$ = 2; ^{13}C NMR: 19.4, 64.45, 77.4, 93.1, 116.9, 118.1, 170.6.

(E)-1-Acetoxy-11,13-tetradecadien-9-yne. A solution of $ZnBr_2$ [24.8 g (0.11 mol)/100 ml THF] was added to a 1 M THF solution of vinyl magnesium bromide (100 ml, 0.1 mol) cooled at 0°C. The reaction mixture was stirred at 20°C for 30 min, and after cooling to 0°C, 0.7 g (0.6 mmol) of $Pd(PPh_3)_4$ in THF (20 ml) followed by 6 g (0.02 mol) of bromoynene were added. The stirred mixture was allowed to warm to room temperature for 12 hr and then hydrolyzed by H_2SO_4 solution (1 N). After extraction with ether and evaporation of the solvent the crude mixture was passed through a small packed silica gel column (eluting with cyclohexane). The solvent was evaporated and the residue distilled to afford 3.7 g of acetoxydienyne (75% yield); *E* purity (96%); bp 115°C/0.1 Torr; *m/z*: 248 (M^+, 3%), 91 (100%); IR: 2250, 1740, 1620, 1245, 1000, 940, 905, 840, 745, 720, 700; ^{1}H NMR: 1.33 (m, 12H), 2.02 (s, 3H), 2.30 (m, 2H), 4.04 (t, 2H), 5.09 (dd, H^{14}), 5.21 (dd, $H^{14'}$), 5.59 (d, H^{11}), 6.25 (dd, H^{12}), 6.45 (ddd, H^{13}) $J_{H^{13}/H^{14'}}$ = 16.5, $J_{H^{11}/H^{12}}$ = 14.4, $J_{H^{12}/H^{13}}$ = 9.9, $J_{H^{13}/H^{14}}$ = 9, $J_{H^{14}/H^{14'}}$ = 2; ^{13}C NMR: 64.5, 79.6, 93.4, 112.7, 118.4, 136.4, 140.9, 171. Anal. calcd. for $C_{16}H_{24}O_2$: C, 77.38; H, 9.74. Found: C, 77.33; H, 9.62.

(E)-11,13-Tetradecadien-9-yn-1-ol. This product was prepared by saponification of the corresponding acetate with 2 N KOH in MeOH; Crude yield (98%); *E* purity (96%); *m/z*: 206 (M^+, 4%), 91 (100%); IR: 3350, 2200, 1620, 1580, 1000, 940, 905, 840, 740, 695; ^{1}H NMR: 1.34 (m, 12H), 2.32 (m, 2H), 3.0 (m, 1H), 3.57 (t, 2H), 5.08 (dd, H^{14}), 5.20 (dd, $H^{14'}$), 5.58 (d, H^{11}), 6.24 (dd, H^{12}), 6.45 (ddd, H^{13}) $J_{H^{13}/H^{14'}}$ = 16.5, $J_{H^{11}/H^{12}}$ = 14.4, $J_{H^{12}/H^{13}}$ = 10.8, $J_{H^{13}/H^{14}}$ = 9, $J_{H^{14}/H^{14'}}$ = 2. ^{13}C NMR: 62.5, 79.6, 93.5, 112.7, 118.4, 136.3, 140.8.

(Z,E)-9,11,13-Tetradecatrien-1-ol. A mixture of dienynol (2.9 g, 0.014 mol), 1-propanol–water (1/1) (600 ml), zinc powder (200 g), and potassium cyanide (10.4 g, 0.16 mol) was stirred at room temperature for 24 hr under argon in the dark. The reaction product was filtered through celite, and the filtrate was extracted with Et_2O. After evaporation of the solvent, 2.6 g of trienol were obtained (90% crude yield); *ZE* purity (98%); *m/z*: 208 (M^+, 11%), IR: 3340, 1620, 1570, 1000, 935, 895, 740, 720, 695; ^{1}H NMR: 1.33 (m, 12H), 1.99 (m, 1H), 2.16 (m, 2H), 3.60 (t, 2H), 5.07 (dd, H^{14}), 5.20 (dd, $H^{14'}$), 5.46 (dt, H^9), 6.00 (ddt, H^{10}), 6.19 (dd, H^{12}), 6.41 (ddd, H^{13}), 6.51 (dd, H^{11}) $J_{H^{13}/H^{14'}}$ = 16.8, $J_{H^{11}/H^{12}}$ = 14.6, $J_{H^9/H^{10}}$ = 11, $J_{H^{10}/H^{11}}$ = 10.6, $J_{H^{12}/H^{13}}$ = 10.6,

$J_{H^{13}/H^{14}} = 10$, $J_{H^8/H^9} = 7.7$, $J_{H^{14}/H^{14'}} = 2$, $J_{H^8/H^{10}} = 1.5$; ^{13}C NMR: 62.4, 116.6, 128.4, 128.6, 132.9, 133.2, 137.2. Anal. calcd. for $C_{14}H_{24}O$: C, 80.71; H, 11.61. Found: C, 80.94; H, 11.45.

(Z,E)-1-Acetoxy-9,11,13-tetradecatriene. Ac_2O, 1.4 ml (15 mmol), was added to a stirred mixture of 1 g (5 mmol) of alcohol and 1.2 ml of pyridine. Stirring was maintained for 3 hr at room temperature, and the mixture was poured on crushed ice. After extraction with ether and evaporation of the solvent, 1.1 g of acetate were obtained (90% crude yield); *ZE* purity (98%); *m/z*: 250 (M^+, 10%), 79 (100%); IR: 1745, 1235; ^1H NMR: 1.32 (m, 12H), 2.02 (s, 3H), 2.16 (m, 2H), 4.04 (t, 2H). Data of the double-bond system are the same as those of trienol. ^{13}C NMR: 64.6, 116.85, 128.7, 128.8, 133.2, 133.3, 137.4, 170.8. Anal. calcd. for $C_{16}H_{26}O_2$: C, 76.75; H, 10.47. Found: C, 76.50; H, 10.36.

(Z,E)-9,11,13-Tetradecatrien-1-al. To a solution of alcohol, 1 g (5 mmol) in CH_2Cl_2 (20 ml), was added 5.6 g (15 mmol) of PDC (pyridinium dichromate). The reaction mixture was stirred at room temperature for 8 hr, mixed with Et_2O, and filtered through a small column packed with Florisil to give, after evaporation of solvents, the desired compound, 0.9 g (90% crude yield); *ZE* purity (98%); *m/z*: 206 (M^+, 6%), 70 (100%); IR: 2700, 1720; ^1H NMR: 1.31 (m, 10H), 2.15 (m, 2H), 2.40 (td, 2H), 9.72 (t, 1H) ($J = 1.75$). Data of the double-bond system are identical to those of trienol. ^{13}C NMR: 43.8, 116.7, 128.5, 128.6, 133.0, 133.15, 137.2, 203.3. Anal. calcd. for $C_{14}H_{22}O$: C, 81.50; H, 10.75. Found: C, 81.71; H, 10.91.

Other Chemicals. The dienic compounds and (*Z*)-9,11-tetradecenyn-1-al (Z9,Yne11-14:Ald) were prepared as described in Ramiandrasoa and Tellier (1990). The other geometric isomers of trienic compounds, used in the identification work, were synthesized as described in Tellier et al. (1991) and Tellier (1991).

Electrophysiology

Electroantennograms were recorded on male *S. cecropia* secured in a styrofoam block. After a few antennal segments had been snipped off, the distal end of one antenna was inserted into the tip of the recording electrode. The neutral electrode was implanted in the neck of the insect. Both electrodes were glass capillaries filled with Roeder's saline (NaCl: 9.0 g/liter; KCl: 0.2 g/liter; glucose: 4.36 g/liter). Electrodes were connected to a WPI preamplifier through chloridized silver wires. The filtered signal (DC to 300 Hz) was fed into an oscilloscope and monitored directly on the screen.

To stimulate the antenna, air was puffed (0.5 sec; 1.5 liters/min) onto the antenna through a glass cartridge containing a piece of filter paper impregnated with 500 ng of synthetic compound. Ten compounds were tested, including

those found in gland extracts of female *S. cecropia*, and a number of analogs. All were synthesized in the laboratory, with an isomeric purity better than 98%. The response profiles to synthetic compounds were recorded on 10 males (five replicates only for the trienic aldehyde, acetate, and alcohol that had not been synthesized at the beginning of the EAG screening).

Single sensillum recordings were carried out on excised antennae, using the tip recording technique (Kaissling and Thorson, 1980; Lucas and Renou, 1989). The base of the antenna was inserted in a glass microelectrode filled with hemolymph saline (Kaissling and Thorson, 1980). The tip of a few sensilla trichodea were cut off with sharpened forceps, and the recording electrode, filled with receptor lymph saline, was slipped over the cut end of a single hair. The action potentials were filtered (150–5000 Hz), amplified ($\times 1000$), and recorded on an audio tape recorder (Revox B77). The recordings were then played back and digitized, with a 10-kHz conversion rate, by an A/D converter board (Dash 16 of Metrabyte) integrated in a PC microcomputer. The responses were analyzed using a specific program written in Asyst (Macmillan Software Company) language (Lucas and Renou, 1989).

The outlet of a Y-shaped glass tube was positioned 2 cm from the antenna, and a flow of humidified pure air (1.5 liters/min) was continuously blown over the antenna through one of the lateral branches. The stimulation cartridge consisted of a Pasteur pipet containing a piece of filter paper (0.5 cm^2) on which hexane extracts or hexane solutions of compounds were deposited. This cartridge was introduced in the main branch of the Y tube. Stimulation was achieved by blowing a puff of air (0.5 sec; 0.5 liters/min) through the cartridge. The same compounds as those used for EAG were assayed on 14 sensilla trichodea belonging to six different male moths.

Olfactometric Tests

The availability of tens of living pupae in the spring of 1989 enabled us to develop a laboratory-based bioassay of pheromones. As we had insufficient time to check the optimum conditions for wind-tunnel tests, we chose a dynamic, linear olfactometer (Sower et al., 1973; Zagatti et al., 1987), consisting of a glass tube (70 cm long, 3.5 cm diameter). Air was humidified by bubbling into water and heated to 25°C, then flowed into the tube at 1.5 liters/min. The olfactometer was situated in a room heated at 25°C and lighted by three incandescent red bulbs (40 W each).

The test started just at the beginning of the scotophase and lasted 1 hr. Eight virgin males (1 or 2 days old) were placed at the downwind end of the tube. Then the blend of synthetic compounds to be tested was deposited (1 μl) on a glass applicator at the upwind end of the tube. The males were scored during 3 min according to whether they exhibited any of the following behavior

patterns: (1) wing fanning, (2) orientation toward the source by walking through one half of the olfactometer length, and (3) contact with the source of attractant with or without copulation attempts. These behavior patterns always occurred in this order in a hierarchical sequence. Each male was tested only once, and 112 males were tested in 14 sets.

To test synthetic attractants, the compounds were diluted in hexane (Carlo Erba Analyticals RPE) and the blends were prepared to obtain the doses (in 1 μl hexane) shown in Table 1. These six blends were qualitatively the same as those used during the 1989 field trapping experiment in San Alberto (Table 2). In addition, the effect of natural pheromone was assayed by clipping off the pheromone glands of two calling females and crushing them immediately to be tested on the glass applicator.

The results were analyzed by a 7 $\times$ 2 chi-square test for global heterogeneity ($P < 0.05$), and then all blends or female glands were compared pairwise using a 2 $\times$ 2 chi-square test. As this method multiplies the degrees of freedom, the threshold of significance for the pairwise comparisons was fixed at $P < 0.01$, although the overall significance of the test was $P < 0.05$. The last step of the behavioral sequence (contacts with source) was not analyzed, due to a small number of responses.

Field Trapping

The first field-trapping experiments were conducted in August–October 1989 in San Alberto (estate of Indupalma S.A.). In the first part of this experiment, the efficiency of pheromone trapping was studied by using traps baited with virgin females. A trap containing two virgin females was hung 1.5 m above the ground on a palm tree. This palm tree was examined every week to check the number of living pupae on leaf number 17 and the number of eggs on 10 leaflets of leaf number 17. This is a standard method for monitoring populations of *S. cecropia* (Genty et al., 1978). Three traps were used simultaneously and the palm trees containing them subsequently sampled. The traps were made from plastic cans (1 gallon vol) from which two windows (10 $\times$ 5 cm) were cut. A small wire mesh cage was hung inside the trap in front of the windows, and the bottom of the trap was filled with water and detergent. Two 1-day old virgin females were held in the cages and replaced every two days. The trap catches were recorded every day.

The second part of this experiment was conducted simultaneously to compare the attractancy of synthetic chemicals. These chemicals were synthesized in the laboratory with an isomeric purity greater than 98%. Six blends were compared (Table 2). All tested blends were diluted in 1 ml hexane and loaded on methanol extracted rubber septa (SNL, Paris, France). The trap design was the same as in the virgin female trapping experiment, except that the pheromone

TABLE 1. RESULTS OF OLFACTOMETRIC TESTS WITH MALE *S. cecropia* AND DIFFERENT SYNTHETIC BLENDS

Test	Compounds (ng)					*N*	Wing fanning[a] (%)	Orientation to source[a] (%)	Contacts with source[b] (%)
	Z9,E11,13-14:Ald	Z9,E11-14:Ald	Z9,E11,13-14:Ac	Z9,E11-14:Ac	Z9,Yne11-14:Ald				
1	50	10	30	10		32	62.5a	31.3ab	25
2		10	30	10	50	16	31.3ab	0b	0
3	50		30			8	37.5ab	0b	0
4		10		10		8	0b	0b	0
5	50	10				24	62.5a	33.3a	16.7
6	50			10		8	37.5ab	12.5ab	12.5
7	Two crushed glands of virgin females					16	62.5a	37.5a	18.8

[a]The results are expressed as percentages of males showing the behavior. In the same column, numbers followed by the same letter are not significantly different (global heterogeneity tested as a 7 × 2 chi-square analysis at $P = 0.05$, then 2 × 2 single comparisons at $P = 0.01$).

[b]Statistical analysis not available for this level of response.

TABLE 2. TOTAL CATCHES OF ADULT *S. cecropia* IN TRAPS BAITED WITH DIFFERENT SYNTHETIC BLENDS FROM AUGUST 26 TO SEPTEMBER 27, 1989 (SAN ALBERTO, CENTRAL COLOMBIA)

Blend	Compounds (μg)					Male catches[a]	Female catches[a]
	Z9,E11,13-14:Ald	Z9,E11-14:Ald	Z9,E11,13-14:Ac	Z9,E11-14:Ac	Z9,Yne11-14:Ald		
SC1	250	50	150	50		28c	22a
SC2		50	150	50	250	19c	22a
SC3	250		150			29c	15a
SC4		50		50		16c	13a
SC5	250	50				88b	24a
SC6	250			50		20c	15a
2 virgin females[b]						458a	24a

[a] In the same column, numbers followed by the same letter are not significantly different at $P = 0.05$ [one-way ANOVA on seven treatments and three replicates after transformation of data to $\sqrt{(x + 0.5)}$ and Newman-Keuls test]. The differences between male catches and female catches in the same row are not significant ($P > 0.05$), except for SC5 and for the virgin female traps (Wilcoxon's randomization test for matched pairs).

[b] See Figure 3 for chronological data.

TABLE 3. TOTAL CATCHES OF ADULT *S. cecropia* IN TRAPS BAITED WITH DIFFERENT SYNTHETIC BLENDS FROM JUNE 6 TO JUNE 20, 1992 (TUMACO, COLOMBIA)

Blend	Compounds (μg)			Male catches[a]	Mean catch/day/trap	Female catches
	Z9,E11,13-14 : Ald	Z9,E11-14 : Ald	Z9-14 : Ald			
SC5	250	50		27b	0.6	0
SC7	250			0c	0	0
SC8		50		0c	0	0
SC9	250	50	10	9bc	0.2	0
SC10	250	250		14bc	0.3	0
SC11	1000	200		113a	2.7	0
SC12	250	5		0c	0	0

[a]Numbers followed by the same letter are not significantly different at $P = 0.05$ (two-way ANOVA on three blocks, after transformation of data to $\sqrt{(x + 0.5)}$ and Newman-Keuls test).

dispensers were hung inside the trap in front of the windows. The trap catches were recorded daily for one month, with three replicates of each blend. The pheromone dispensers were changed once, 18 days after the beginning of the experiment.

A second trapping campaign was scheduled in June 1992 in Tumaco (Nariño, south of Colombia) in a 3500-ha plantation (estate of Palmas de Tumaco Ltda.). Synthetic chemicals, dispenser, and trap design were the same as in San Alberto. Seven blends were compared (Table 3) with three replicates in a completely randomized block design. The trap catches were recorded daily for two weeks.

The results were submitted to one-way (San Alberto) or two-way (Tumaco) analysis of variance on data transformed by $\sqrt{(x + 0.5)}$ to reach homoscedasticity. The means were then compared by Newman-Keuls test with $P < 0.05$ (Dagnélie, 1975). As some traps caught both males and females, the differences between male and female catches were compared using Wilcoxon's randomization test for matched pairs (Siegel, 1956).

RESULTS

Pheromone Idenification

GC-MS analyses of the 557, 684, and 759 female gland extracts revealed the same chromatographic pattern. In addition to saturated and unsaturated hydrocarbons and fatty acids, GC-MS data (in electron impact mode) indicated

two 14-carbon aldehydes, one of them diunsaturated [*m/z* 41, 55, 67 (100%), 82, 95, 105, 208 ($M^+\cdot$; confirmed by CI-NH_3 with ions at *m/z* 209 and 226)] and the other triunsaturated [*m/z* 41, 55, 67, 79 (100%), 80, 91, 107, 206 ($M^+\cdot$; ions at *m/z* 207 and 224 in CI-NH_3)]. The corresponding 14-carbon acetates were also identified [one of them was diunsaturated with ions at *m/z* 43, 55, 67 (100%), 79, 82, 93, 110, 121, 192 (M-CH_3COOH) and 252 ($M^+\cdot$; ions at *m/z* 253 and 270 in CI-NH_3) and the other triunsaturated with ions at *m/z* 43, 55, 67, 79 (100%), 80, 91, 105, 119, and 250 ($M^+\cdot$; ions at *m/z* 251 and 268 in CI-NH_3)]. The diunsaturated aldehyde was identified by chemical ionization analysis using isobutane to determine the position of the double bonds (Einhorn et al., 1985). The mass spectrum exhibited various molecular species: $M^+\cdot$ (*m/z* 208, 22%), $(M + C_4H_9)^+$ (*m/z* 265, 4%), MH^+ (*m/z* 209, 91%), $(MH\text{-}H_2O)^+$ (*m/z* 191, 39%), and revealed the presence of abundant ions at *m/z* 71 (33%), 169 (100%), and 151 (60%), which were clearly related to a 9,11 position of the diethylenic system. The stereochemistry was assigned to (*Z,E*)-9,11 by comparison of the GC retention times with standards. Data on the trienic system were completely lacking at the beginning of our work. The mass spectrum could be assigned either to a triene or to a triple bond, Δ9,Yne11-enyne. The triene hypothesis of a conjugated system with a terminal double bond was suggested by comparing the mass spectrum of the natural product with spectra of several chemicals sharing the same trienic motif, already prepared in our laboratory as reaction intermediates during studies on moth pheromones. Finally, all possible isomers of (Δ,Δ)-9,11,13 and Δ9,Yne11 14-carbon aldehydes and acetates were synthesized, and comparison of GC retention times and mass spectra proved that the natural products were (*Z,E*)-9,11,13-tetradecatrienal and (*Z,E*)-9,11,13-tetradecatrienyl acetate. The extracts of pheromone glands of *S. cecropia* contained: (*Z,E*)-9,11-tetradecadienal (*Z*9,*E*11-14:Ald; 11%), (*Z,E*)-9,11,13-tetradecatrienal (*Z*9,*E*11,13-14:Ald; 67%), (*Z,E*)-9,11-tetradecadienyl acetate (*Z*9,*E*11-14:Ac; 5.5%) and (*Z,E*)-9,11,13-tetradecatrienyl acetate (*Z*9,*E*11,13-14:Ac, 16.5%).

Electrophysiology

The EAG screening demonstrated that the two aldehydes found in the female gland extracts were active on the antenna of male *S. cecropia* (Figure 2). The most active compound was the triene aldehyde, *Z*9,*E*11,13-14:Ald, which elicited an EAG response of 1.9 ± 0.7 mV (mean ± 95% confidence interval). The dienic aldehyde, *Z*9,*E*11-14:Ald, also elicited high-amplitude EAG (1.5 ± 0.5 mV). Responses to other compounds assayed, and in particular to the two acetates identified in the gland extracts, were all inferior to 0.7 mV.

The activity of 14 sensilla trichodea was recorded from six different males in response to 10 pheromone compounds and pheromone analogs. Sensilla trichodea could be separated into four different groups based on the response profiles of their sensory neurons (Figure 2). Sensilla 1–6 presented a firing

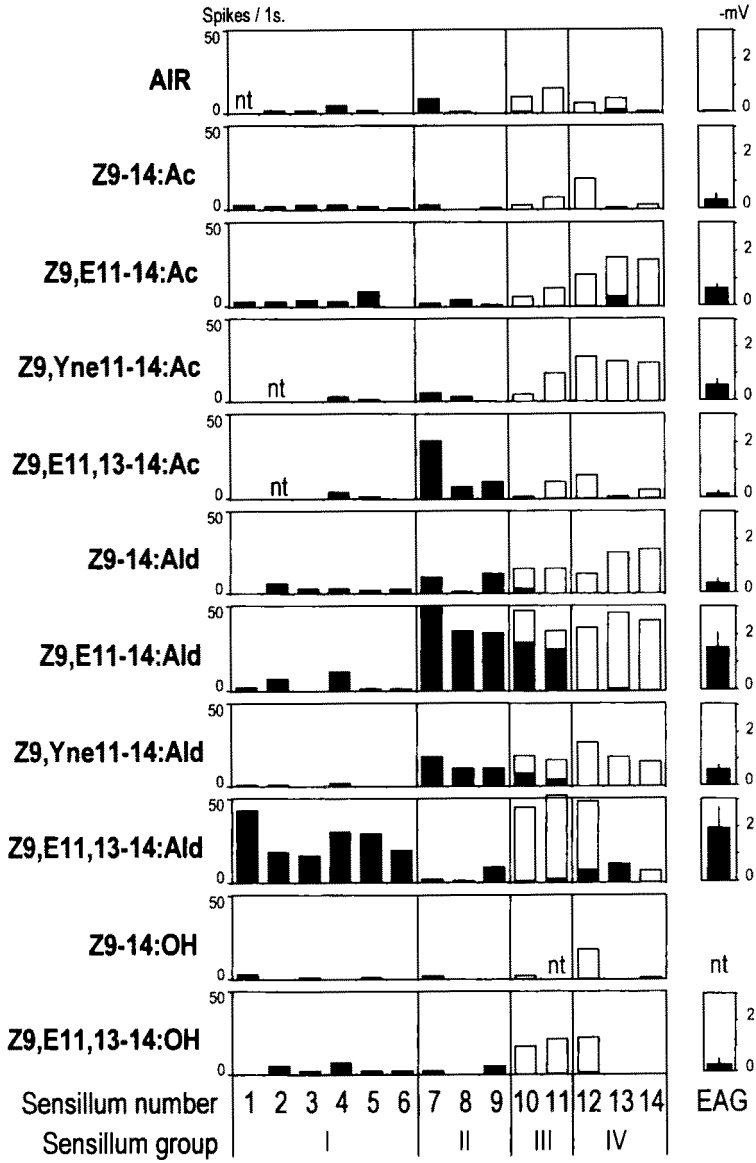

FIG. 2. Electroantennograms (EAG) and single sensillum recordings obtained from male *Stenoma cecropia* antennae stimulated by synthetic compounds. EAG are expressed as the mean (in millivolts) of 5–10 replicates. The vertical bars represent the 95% confidence interval of the mean. The single sensillum recordings were obtained from 14 sensilla trichodea belonging to six different male moths. Black bars: A cell activity; open bars: B cell activity (groups III and IV). Name of compounds were abridged as follows: Z9-, geometry and position of the double bond; Yne11-, triple bond; -14, chain length; OH, Ald, Ac, alcohol, aldehyde, and acetate, respectively; nt: compound not tested.

activity consisting of a single amplitude class of action potentials. Their firing activity was stimulated by Z9,E11,13-14:Ald. Responses to other compounds were not significant when compared to the air control. A second group, sensilla 7-9, presented a single class of action potentials and responded to the other EAG-active compound, Z9,E11-14:Ald. These sensilla were less specific than group I sensilla since they also weakly responded to Z9,E11,13-14:Ac and to Z9,Yne11-14:Ald. The firing activity of group III (sensilla 10 and 11) and group IV (sensilla 12-14) clearly consisted of two amplitude classes of action potentials, indicating the presence of at least two receptor neurons. In groups III and IV, the cells firing high-amplitude action potentials, up to 3 mV, are referred to as the A cells. Action potentials emitted by the other cells, referred to as B cells, were much smaller (50% of the amplitude of A cell action potentials). In group III sensilla, A cells clearly responded to Z9,E11-14:Ald and B cells to Z9,E11,13-14:Ald. The response profiles of sensilla from group IV were characterized by strong responses from B cells to Z9,E11-14:Ald and to a lesser exent to Z9,E11-14:Ac, Z9,Yne11-14:Ac, Z9-14:Ald and Z9,Yne11-14:Ald. In two out three of these sensilla, A cells responded weakly to Z9,E11,13-14:Ald.

Olfactometric Tests

The results are shown in Table 1. It appeared that two glands of virgin females were able to evoke wing fanning and orientation toward the odor source in male moths. The percentage of moths making contact with the source was very low (18.8%). Two blends of synthetic compounds evoked the same responses by virgin males as did female glands: a blend of Z9,E11-14:Ald, Z9,E11,13-14:Ald, Z9,E11-14:Ac, and Z9,E11,13-14:Ac and a blend of Z9,E11-14:Ald and Z9,E11,13-14:Ald.

Field Trapping

The results of the first trapping experiment (Figure 3) showed that the population levels were very high. The number of living pupae per leaf reached 30 at the beginning of the experiment. The emergence of adults, as shown by the decrease of pupa counts, occurred during the first days of September. By this time the number of unhatched eggs had increased and reached a maximum on September 8. These data suggest that the population of sexually active females was at its highest between September 3 and September 15, 1989.

The attractiveness of virgin females as bait was not correlated with these data. The number of males caught in traps was very small during the first two weeks of September, started increasing as late as September 18, and reached a maximum of 37 males/day/trap on September 20. The three female traps also

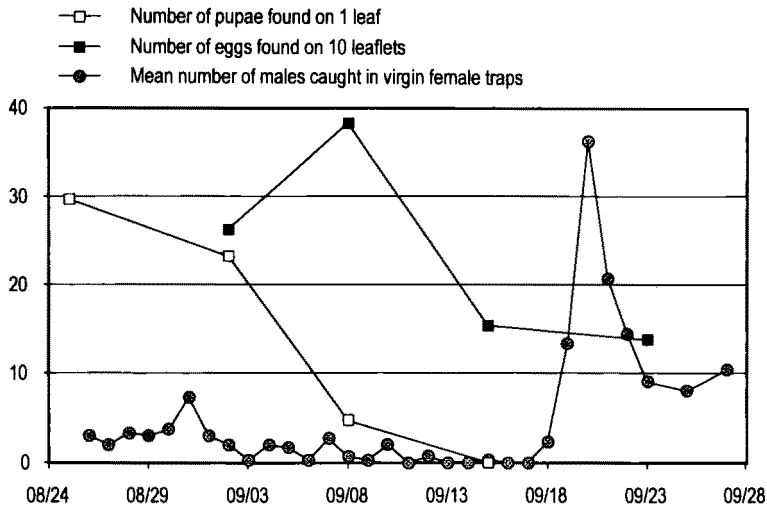

FIG. 3. Chronological catches of *Stenoma cecropia* males obtained with traps baited with two virgin females (mean of three traps) from September 25 to October 27, 1989 (San Alberto, central Colombia). The population levels were estimated by counting living pupae on leaf number 17 and counting unhatched eggs on 10 leaflets (of leaf number 17) on the trees from which the traps were hung (mean of three counts). The scale of the *y* axis is the same for the three curves.

caught females regularly throughout the month the experiment lasted, with an average of 0.25 females/day/trap.

The other part of this experiment was carried out at the same time and in the same habitat as virgin female trapping. It consisted of a comparison between different synthetic pheromone blends. The results (Table 2) showed that all traps caught female moths (average of 0.2 female/trap/day), with no significant differences between blends. Male and female catches in the same trap were significantly different when the traps were baited with blend SC5 (*Z*9,*E*11–14 : Ald, 50 μg, and *Z*9,*E*11,13–14 : Ald, 250 μg) or virgin females ($P = 0.031$). Blend SC5 caught significantly more males than the other blends but catches with this blend were five times lower than those obtained using the virgin females as bait.

The second trapping campaign was conducted in 1992 with a low *S. cecropia* population level that did not exceed one larva per leaf. Mono- or polyunsaturated aldehydes were tested along with the best blend of the 1989 experiment (SC5). The results (Table 3) showed that only males were caught. Blend SC11 caught four times more males than SC5 (Table 3). It consisted of the same blend of *Z*9,*E*11–14 : Ald and *Z*9,*E*11,13–14 : Ald at a load four times higher than

SC5. When tested separately (SC7 and SC8, Table 3) these aldehydes were not attractive.

DISCUSSION

Sex Pheromone of Stenoma cecropia

Four compounds were identified in the sex pheromone gland extracts of *Stenoma cecropia* females: a dienic and a trienic aldehyde, and the corresponding acetates. The electrophysiological study gave evidence for specific receptors to the aldehydes only. The olfactometric tests showed a similar level of response to female glands and to a blend containing the four synthetic compounds in a ratio close to that of female extracts. However, removal of the two acetates did not alter the response of males. In field-trapping experiments, blends containing the dienic and trienic aldehydes attracted males, whereas a four-component blend, which included the acetates, did not.

From these biological data it can be concluded that (*Z*,*E*)-9,11,13-tetradecatrienal and (*Z*,*E*)-9,11-tetradecadienal are components of the sex pheromone of *Stenoma cecropia*. The corresponding acetates do not seem to be involved in the pheromone communication system. They are probably precursors of the aldehydes in a way similar to the biosynthetic pathway described in *Choristoneura fumiferana*. In this tortricid moth, the pheromone components (*E*- and *Z*-11-tetradecenal) are biosynthesized de novo via Δ-11-tetradecen-1-ol and Δ-11-tetradecen-1-ol acetate. The amounts of Δ-11-tetradecen-1-ol acetate in female glands of *C. fumiferana* vary during the daily cycle as this acetate is used as a storage precursor of the pheromone and converted into the corresponding aldehyde just before pheromone emission (Morse and Meighen, 1984, 1986).

Traps baited with the best attractants in the present work caught fewer insects than traps baited with virgin females. This may mean that at least one component of the pheromone of *S. cecropia* remains unknown.

(*Z*,*E*)-9,11,13-Tetradecatrienal has been identified as a pheromone component of the carob moth *Ectomyelois ceratoniae* (Baker et al., 1989), which belongs to the family Pyralidae, a family not closely related to Elachistidae. This compound was also identified in another *Stenoma* species, *Stenoma decora*, a major pest of cocoa in Brazil (D. R. Hall, personal communication).

The phylogenetic relationships between families of the Gelechioidea remain puzzling. Minet (1989) included in the Elachistidae several genera formerly thought to belong to the Oecophoridae. Some of them are listed in a recent review of sex attractants (Arn et al., 1992): *Ethmia* spp. (Elachistidae: Ethminae), *Depressaria olerella* and *Agonopterix* spp. (Elachistidae: Depressariinae). None of them use aldehydes as sex attractants. Instead they use monoenic acetates or alcohols with chain lengths of 10–14 carbons. Thus, the chemotaxonomy

of sex pheromones does not seem to be promising for clarifying the status of this group.

Influence of Insect Density

The results of the field-trapping experiments raised questions about the influence of the population density on the effectiveness of sexual trapping. During the 1989 season, the population levels were very high. It can be seen in Figure 3 that the maximum number of sexually active females occurred between September 3 and September 15. The number of males caught during this period by the virgin female traps was very small (33 out of a total catch of 458). It began to increase after September 18, at a time when few females were likely to be unmated and calling for males.

It is generally admitted that catches in traps baited with synthetic attractants are not correlated with insect population levels at the highest levels of density (Cardé, 1979). To our knowledge, this has not been reported for traps baited with virgin females. These are expected to be very attractive, because the calling period of the caged females lasts 1 or 2 hr whereas the "free" females stop calling as soon as they mate. The lack of male catches during the early part of our experiment with *S. cecropia* suggests that the insects used other mate-searching systems than pheromone orientation when density was high. The movements of many moths in the vicinity of the traps may explain the number of drowned females (Table 2). It may also result in sexual encounters and subsequent matings.

The monitoring of pest populations with pheromones has now become a routine technique for plant protection in temperate countries. It is not yet widespread in the tropics (Zagatti, 1993), where different pest characteristics may result in a different approach to pest management programs.

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ALLELOPATHIC POTENTIAL OF WELL WATER FROM *Pluchea lanceolata*-INFESTED CULTIVATED FIELDS

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Abstract—*Pluchea lanceolata*, an allelopathic perennial weed, has an extensive deep root and rhizome system. The objective of the present study was to determine the allelopathic potential of well water collected from weed-infested cultivated fields. Results indicate that well water recovered from *P. lanceolata*-infested cultivated fields inhibited the shoot growth of pea, chick pea, mustard, and wheat under greenhouse conditions. Two phenolic compounds, phenol and phloroglucinol, were isolated and identified from collected well water using UV spectroscopy. The allelopathic potential of the aqueous extract of the two compounds was determined by growth experiments with 10^{-4} M solutions of the compounds. As a consequence of repeated irrigation with well water from *P. lanceolata*-infested fields, higher levels of phenolics can accumulate in the soil, which may contribue to increased interference to crop plants. The present study is of significance since it cautions on the use of well water for irrigating cultivated fields infested with the perennial allelopathic weed (*P. lanceolata*) with dense subterranean systems and emphasizes the importance of controlling such weeds in cultivated fields.

Key Words—*pluchea lanceolata*, allelopathy, weed, phenol, phloroglucinol, well water.

INTRODUCTION

Pluchea lanceolata (DC) C. B. Clarke (Asteraceae) is a perennial weed of semiarid regions of India (Inderjit and Dakshini, 1990). *P. lanceolata* achieves its success by inhibiting the growth and establishment of crop plants through water-soluble phenolics released to the soil (Inderjit and Dakshini, 1990, 1992b).

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Farmers plow the above-ground parts of the weed while preparing fields for cultivation, and irrigation results in leaching of water-soluble phenolics into the soil (Inderjit and Dakshini, 1994b, 1996). The extensive subterranean roots and rhizomes (up to 80 ft deep) leach water-soluble phenolics to the soil (Inderjit and Dakshini, 1994c). Growth of *P. lanceolata* results in availability of water-soluble phenolics throughout the year. A flavonone (hesperidin), a dihydroflavonol (taxifolin 3-arabinoside), a flavonol (quercitrin), an isoflavonoid (formononetin 7-*O*-glucoside), and other phenolics, such as chlorogenic acid, phloroglucinol, and simple phenol, isolated from *P. lanceolata*-infested cultivated soils are potent allelochemicals (Inderjit and Dakshini, 1991, 1992a, 1994c, 1995a).

Irrigation of cultivated fields with well water is a common practice in northern India. Because of the extensive and deep root and rhizome system of *P. lanceolata* and its potential to leach water-soluble phenolics, we hypothesized that phenolics released by the weed may percolate through the soil profile to groundwater level. Thus with each irrigation, the weed not only cycles phenolics but the concentration of phenolics may also increase. The establishment of the relationship between the phenolics released by *P. lanceolata* and phytotoxicity of well water in the field should be of practical value in demonstrating strategies to control the weed's effect on crops. The objectives of the present study were: (1) to study the allelopathic potential of well water collected from fields infested with *P. lanceolata*; (2) to isolate, identify, and quantify the phenolic constituents responsible for the observed allelopathic potential; and (3) to test the allelopathic potential of isolated phenolic compounds.

METHODS AND MATERIALS

The cultivated fields, infested with the weed *P. lanceolata*, were irrigated with water from deep wells (50–75 ft) installed in the fields for 20–25 years (28°38′N, 77°12′E). Each well is a lined shaft sunk in the fields, and serves approximately 1 *ha* of cultivated land. The fields had been infested with the weed for 20 years. Water from three wells installed in these fields was collected in glass containers (Borosil) and refrigerated at 20°C. Additionally, well water from fields not infested with *P. lanceolata* was also collected and served as the controls. No *P. lanceolata* occurred in control fields based upon observations that revealed no *P. lanceolata* visible above the ground, and no roots or rhizomes of *P. lanceolata* were observed after digging the soils up to 4 ft depth.

Both weed-infested and weed-free cultivated fields had been subjected to similar agricultural practices. The surface texture of the soil was sandy loam (coarse sand, 6%; fine sand, 74%; silt, 9% and clay, 11%). The main crops cultivated in this region are radish (*Raphanus sativus*), wheat (*Triticum aesti-*

vum), mustard (*Brassica juncea*), chick pea (*Cicer arietinum*), and tomato (*Lycopersicon esculentum*). Common weeds in these areas are Mexican pricklepoppy (*Argemone mexicana*), common lambsquarters (*Chenopodium album*), field bindweed (*Convolvulus arvensis*), white sweetclover (*Melilotus alba*), calotrope (*Calotropis procera*), onionweed (*Asphodelus tenuifolius*), and sowthistle (*Sonchus arvensis*). The climate of this region is semiarid with a marked hot and dry summer (May–June, temperature 25–45°C), a dry and cold winter (December through mid-February, temperature 4–20°C), and the rainfall (July–September) is moderately low for India (600–1200 mm).

Cultivated fields were heavily infested with *P. lanceolata* (density, 15.9 $\pm$ 4.1 plants/m^2). The pH of the well water (7.7) from weed-infested fields ($N = 26$) was significantly ($P < 0.01$) lower than that of well water from weed-free cultivated fields (8.4).

Evaluation of Well Water for Allelopathic Response. The experiments were designed to study the effect of well water on the growth and establishment of crop plants. Ten seeds of radish (var. pusa chetaki), mustard (var. CV PR45), wheat (var. HD-2923), chick pea (var. H-208), tomato (var. pusa ruby), and turnip (*Brassica rapa* var. purple white top) were sown 2.5 cm deep in clay pots (1500 cm^3) filled with sandy loam soil. The soil had the following characteristics: pH, 7.87 $\pm$ 0.37; electrical conductivity, 231.5 $\pm$ 26.6 μmho/cm; organic matter, 5.54 $\pm$ 1.4 g/kg; organic nitrogen, 38 $\pm$ 7.4 mg/100 g; phosphate, 7.9 $\pm$ 3.2 mg/100 g; Ca, 910 $\pm$ 98.7 mg/100 g; Na, 23.7 $\pm$ 9.88 mg/100 g; Mg, 28.03 $\pm$ 4.08 mg/100 g; K, 33.8 $\pm$ 10.1 mg/100 g; and Zn, 0.300 $\pm$ 0.130 mg/100 g. These pots were kept in an experimental garden of the Department of Botany, University of Delhi. The pots were irrigated daily with 500 ml of well water from weed-infested fields (identified as treatments) or with well water from weed-free fields (identified as controls). A random block design was replicated three times (Panse and Sukhatme, 1967). The average day–night temperature was 25/14°C with average light conditions of 403 $\mu E/m^2/sec$. The emergence of the plumule from the soil by the end of the sixth day after sowing was the criterion for germination. Thereafter, the shoot length was recorded for three weeks. Data were analyzed through ANOVA.

Isolation, Identification and Quantification of Allelopathic Substances. Two liters of well water from weed-infested and weed-free fields were concentrated under vacuum and residues were extracted with 10 ml of methanol. These extracts were chromatographed on Whatman No. 3 (46 $\times$ 57 cm) paper and developed through descending chromatography using butanol–acetic acid–water (BAW, 4:1:5, upper phase) according to Harborne (1973). The developed chromatograms were scanned under UV prior to and after NH_3 treatment and the bands marked.

Each band was eluted in methanol, loaded again separately on Whatman No. 3, washed with distilled water (DW), and finally extracted with methanol.

Care was taken to avoid loss of the compounds during washing and elution. To confirm the purity of these compounds, each compound was spotted on Whatman No. 1 chromatographic paper and run in four solvents: DW, BAW, 15% acetic acid, and forestal (conc. HCl–acetic acid–water, 3:30:10 by volume) (Harborne, 1973). After confirming the purity of the compounds, each was eluted in methanol for spectral analysis, acid hydrolysis, and acid–base shifts (NaOH, $AlCl_3$, $AlCl_3$-HCl, NaOAc, NaOAc-H_3BO_3), if any, of the parent as well as the hydrolyzed fractions (Harborne, 1973; Van Sumare, 1989). To finally confirm the identification, isolated compounds were cochromatographed with authentic reference compounds and their spectral data matched. Two compounds, phenol and phloroglucinol, were quantified with reference to authentic reference compounds (Sigma, St. Louis, Missouri).

Allelopathic Effect of Compounds. Growth experiments were performed to determine the allelopathic potential of the two isolated compounds. The authentic reference compounds were initially dissolved in methanol, dried under vacuum, and then dissolved in water to a final concentration of 10^{-4} M, which is the lowest recommended effective concentration of allelochemicals used to assess phytotoxicity (Inderjit et al., 1995; Rice, 1984; Williams and Hoagland, 1982). The seeds of commonly grown cultivated crops such as radish (var. pusa chetaki), mustard (var. CV PR45), and tomato (pusa ruby) were selected for growth experiments. Fifty seeds (in triplicate) of the respective crop plants were placed on Whatman No. 1 filter paper moistened with 70 ml of 10^{-4} M solutions, with DW as control and placed in 15-cm-diameter Petri dishes. To maintain uniform moisture status in the Petri dishes, a cotton pad soaked in DW or compounds was placed below the filter paper. Root and shoot lengths were recorded every 24 hr for six days, but seedling lengths recorded on the sixth day are presented in the present study. The experiments were conducted at 25 ± 4°C; 373 μE/m^2/sec fluorescent light with a 16/8 light–dark period. The experiments were completely randomized and replicated three times. Data were pooled and analyzed through ANOVA.

RESULTS

Among the seeds tested, only germination of mustard was affected by well water from *P. lanceolata*-infested fields (Table 1). Compared to irrigation with well water from weed-free fields, shoot growth of mustard (31.7%), pea (61.5%), chick pea (36.7%), and wheat (26.9%) was reduced in plants irrigated with well water from weed-infested field (Table 1).

Two bands appeared on chromatograms loaded with well water from weed-infested fields, while no bands were detected on chromatograms loaded with extracts of well water from weed-free fields. These bands were identified as

TABLE 1. RESPONSE OF CROP PLANTS TO WELL WATER FROM *Pluchea lanceolata*-INFESTED CULTIVATED FIELDS

Crop plants	Response relative to control[a]	
	Germination (%)[b]	Shoot length (%)[c]
Wheat	93	−26.9[d]
Pea	50	−61.5[d]
Chick pea	109	−36.7[d]
Mustard	50[d]	−31.7[d]
Radish	96	+1.65
Turnip	107	−7.9

[a]Well water from weed-free cultivated fields served as the control.
[b]Percent germination relative to control.
[c]Average percent increase (+) or reduction (−) in shoot length in comparison to control.
[d]Treatment means are different from control at $*P < 0.05$.

phenol and phlorglucinol through UV spectral studies (Table 2). The concentration of phloroglucinol in well water was 6.8 ± 0.60 mg/liter, while that of phenol was 2.7 ± 0.21 mg/liter (Table 2).

At a concentration of 10^{-4} M, phloroglucinol significantly ($P < 0.05$) inhibited shoot growth of mustard and tomato, while radish was not affected (Table 3). Phenol significantly ($P < 0.05$) inhibited the root growth of mustard and radish, but tomato was not affected (Table 3). Comparatively, shoot growth was more sensitive to phloroglucinol, while root growth was more sensitive to phenol.

DISCUSSION

The presence of phenol and phloroglucinol in well water and the inhibitory effects of well water from weed-infested fields and of phloroglucinol and phenol on crop plants demonstrate the allelopathic potential of well water from *P. lanceolata*-infested cultivated fields. Growth responses of seeds watered with well water from *P. lanceolata*-infested fields and responses of seeds subjected to two phenolic compounds (Table 3) varied with the seeds tested. Presently, even though the causes of such variation can not be explained, it is likely due to differences in seed size, seed permeability, differential uptake, and metabolism (Williams and Hoagland, 1982), thus leading to allelochemical selectivity.

Inderjit and Dakshini (1994c) isolated phenol and phloroglucinol from *P. lanceolata* roots and its associated soils. Since the weed has subterranean roots and rhizomes up to 80 ft in depth and irrigation in cultivated fields is frequent,

TABLE 2. FLUORESCENCE, UV ABSORPTION AND CONCENTRATION OF COMPOUNDS ISOLATED FROM WELL WATER OF *Pluchea lanceolata*-INFESTED CULTIVATED FIELDS

Compound	Fluorescence UV(UV + NH_3)[a]	Absorption maxima						Concentration (mg/liter)[b]
		MeOH	NaOH	$AlCl_3$	$AlCl_3$-HCl	NaOAc	NaOAc-H_3BO_3	
Phloroglucinol	B(FB)	222	232	221	221	222	221	6.8 ± 0.60
		273	281	272	273	272	272	
			350					
Phenol	B(B)	275	281	278	278	278	276	2.74 ± 0.21

[a] Fluorescence key: B, blue; FB, fluorescent blue.
[b] Values are average of two well samples replicated three times.

TABLE 3. EFFECT OF PHLOROGLUCINOL AND PHENOL (10^{-4} M) ON SEEDLING GROWTH OF CROP PLANTS

Compound	Crop plants	Response relative to control[a]	
		Root (%)	Shoot (%)
Phloroglucinol	Mustard	−9.07	−15.29[b]
	Radish	−3.92	−8.17
	Tomato	−21.75	−44.01[b]
Phenol	Mustard	−25.13[b]	−9.90
	Radish	−24.65[b]	−2.75
	Tomato	−12.06	−12.95

[a]Average percent increase (+) or reduction (−) in seedling length in comparison to control.
[b]Treatment values are different from the control at $P < 0.05$.

leaching of these compounds to groundwater levels is very likely. Inderjit and Dakshini (1994c) reported the concentration of phenol and phloroglucinol in aqueous leachates from *P. lanceolata* roots as 46.5 and 25.5 mg/liter, respectively. While these compounds were not detected from *P. lanceolata*-free cultivated soils, their concentration in *P. lanceolata*-infested soils varied from 5 $\times$ 10^{-3} to 10^{-6} M under field conditions (Inderjit and Dakshini, unpublished data). It is difficult to determine the exact bioactive concentrations of these compounds in soils under field conditions, because the compounds have been determined through the input of leaf leachates, root exudates, decomposing and leaching from plant parts, sorption to seeds and roots, fixation by soil components, and microbial activity (Blum and Shafer, 1988; Cheng, 1995; Dalton et al., 1983; Haider and Martin, 1975; Rice, 1984). Because of the uneven distribution of aboveground and subterranean parts of *P. lanceolata*, the concentration of these two compounds depends on the amount of roots and incorporated leaves present in soil pockets (Liebl and Worsham, 1983). Each irrigation probably contributes to additional leaching of chemicals. Even low concentrations of allelochemicals may significantly influence plant growth (Muller, 1966). Total phenolics of soil were higher in weed-infested cultivated fields (Inderjit and Dakshini, 1994a; 1996). This may be partly the result of frequent irrigation of cultivated fields with well water containing phenolics.

Flavonoids such as quercetin and quercitrin from *P. lanceolata* leaves, and hesperidin, taxifolin 3-arabinoside, formononetin 7-*O*-glucoside, and quercitrin from *P. lanceolata*-infested soils have been reported (Inderjit and Dakshini, 1991, 1992a, 1994c, 1995a). Flavonoids are known to degrade physically, chemically, and/or microbially in the soils yielding simple phenols and phloroglucinol (Chang et al., 1969; Henderson, 1956; Padron et al., 1960; Rice,

1984; Tanrisever et al., 1987). *P. lanceolata* has the potential to contribute phenol and phloroglucinol either through direct leaching or through degradation of flavonoids contributed to the soil by the weed. Although data on length of time phenol and phloroglucinol persist in soil or well water is essential to finally establish their allelopathic potential, it could be argued that the maintenance and periodic replenishment of these compounds in well water from *P. lanceolata*-infested fields is due to the perennial and evergreen nature of the weed. The allelopathic activity of phloroglucinol and phenol are well documented (Inderjit et al., 1995; Rice, 1984, Waller, 1987); however, the presence of phenol and phloroglucinol in well water from an allelopathic weed is demonstrated for the first time.

The lower pH of well water from weed-infested cultivated fields could be due to the presence of phenolics contributed by the plant (Dalton et al., 1983). The present study offers a reasonable explanation for the growth responses of crop plants to well water. Involvement of other factors such as nutrients and soil characteristics were minimized since the same soils were used in both control and treatments. Moisture stress was eliminated through watering each pot daily with well water from weed-infested (treatments) and weed-free cultivated field (controls). Repeated irrigation of cultivated fields with phenolic-loaded well water will not only degrade the quality of groundwater with time, but such a practice could result in making the fields unsuitable for cultivation. The present study is of significance since it serves as a caution on the use of well water for irrigating drawn from fields infested with the perennial allelopathic weed *P. lanceolata*. The results further emphasize the importance of controlling such weeds in cultivated fields.

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WITHIN-PLANT VARIATION IN CONCENTRATIONS OF AMINO ACIDS, SUGAR, AND SINIGRIN IN PHLOEM SAP OF BLACK MUSTARD, *Brassica nigra* (L.) KOCH (CRUCIFERAE)

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Abstract—Although within-plant variation in the nutrient and allelochemical composition of phloem sap has been invoked to explain patterns of host use by phloem-feeding insects, little is known about within-plant variation in phloem chemistry. Here I describe a new technique in which I use the green peach aphid, *Myzus persicae* Sulz., to investigate within-plant variation in the concentrations of chemicals in the phloem sap of black mustard, *Brassica nigra* (L.) Koch (Cruciferae). Relationships between the concentrations of chemicals in aphid diets and honeydew were established using honeydew from aphids fed on artificial diets with known concentrations of amino acids, sucrose, and sinigrin. These relationships were applied to honeydew from aphids fed on different aged leaves of black mustard to estimate the concentrations of the chemicals in phloem sap. Sinigrin concentration was estimated to be high (>10 mM) in phloem sap in young leaves, calling into question the prevailing opinion that phloem sap contains only low concentrations of allelochemicals. High concentrations may function as defenses against sap-feeding herbivores. Within-plant variation in phloem sap composition was high: (1) young leaves had high concentrations of nutrients (216 mM amino acids, 26% sugar) and sinigrin (>10 mM); (2) mature and presenescent leaves had lower concentrations of nutrients (77–83 mM amino acids, 19–20% sugar) and low concentrations of sinigrin (1–2 mM); and (3) senescing leaves had high concentrations of nutrients (199 mM amino acids, 25% sugar) and low concentrations of sinigrin (3 mM).

Key Words—*Myzus persicae*, *Brassica nigra*, phloem sap, glucosinolates, sinigrin, herbivore distributions, plant defense.

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INTRODUCTION

Extensive variation in the nutrient and allelochemical composition of foliage has been documented within individual plants, and this variation has been proposed to explain spatial and temporal patterns of host use by leaf-chewing insects (e.g., Feeny, 1970; Mattson, 1980; Denno and McClure, 1983). Similar arguments have been made to explain host use by phloem-feeding insects (van Emden, 1972; Dixon, 1976; Wink et al., 1982; Whitham, 1983; Wink and Römer, 1986), but almost no information is available about within-plant variability in the chemistry of phloem sap. Phloem sap contains two macronutrients, amino acids and sugars (usually sucrose). Although concentrations of these chemicals in phloem sap have been measured in many plants (e.g., Becker, 1973; and cited in Ziegler, 1975; Zimmerman and Ziegler, 1975), their variability within individual plants has been investigated in only two species: *Salix acutifolia* (Mittler, 1958) and *Lupinus albus* (Pate et al., 1979). Similarly, the presence of allelochemicals in phloem sap is now firmly established (Molyneux et al., 1990; Montllor, 1991). However, within-plant variability in the concentrations of these chemicals in phloem sap has been assessed in only one species, *Lupinus consentinii* (Wink et al., 1982).

A major obstacle to examining within-plant variability in the chemical composition of phloem sap has been the difficulty of obtaining phloem sap in sufficient quantity and purity for chemical analysis. For example, the method that probably yields the purest samples, the severed-stylet technique (Kennedy and Mittler, 1953), suffers from three potentially serious drawbacks (Fisher and Frame, 1984). First, not all plant species exude phloem sap through severed homopteran stylets. Second, even among those species that do, yields are generally small, especially from herbaceous plants. Third, exudation rates can vary greatly depending on tissue age. This last drawback is especially serious, for if parts of a plant cannot be sampled, within-plant variability cannot be meaningfully assessed.

Here I present a new technique for estimating the composition of phloem sap from aphid honeydew. This technique is used to examine the exposure of the green peach aphid, *Myzus persicae* Sulz., to nutrients and allelochemicals in phloem sap of different aged leaves of black mustard, *Brassica nigra* (L.) Koch (Cruciferae). The green peach aphid is a polyphagous herbivore that feeds on phloem sap (Pollard, 1973; but see van Emdem et al., 1969), and black mustard is one of the aphid's host plants. The phloem sap of crucifers contains amino acids (Pate, 1973) and glucosinolates (Weber et al., 1986), a class of sulfur-containing allelochemicals. Natural populations of the aphid on crucifers occur mainly on the lower, senescing leaves of the plants (van Emden et al., 1969), and it has been hypothesized that this pattern results from within-plant

differences in the nutrient and allelochemical composition of phloem sap (van Emden, 1972).

METHODS AND MATERIALS

Aphids. Two clones of the green peach aphid were used. One was initiated from a single asexually reproducing female (virginopara) obtained from a greenhouse population feeding on tree tomato (*Cyphomandra crassicaulis*, Solanaceae) at the University of Michigan Matthaei Botanical Gardens. The clone was maintained for two years in the laboratory on radish seedlings (var. Red Devil B, Ferry-Morse Seed Co., Modesto, California) at room temperature under long day conditions (18L:6D). Voucher specimens are deposited at the Illinois State Natural History Survey, Champaign, Illinois. A second clone was obtained from a laboratory culture at the University of California at Berkeley, where it had been maintained for many years on radish (T. E. Mittler, personal communication.)

Honeydew Collection from Aphids Fed on Artificial Diets. I used chemically defined liquid diets developed for the green peach aphid (Kunkel, 1977; diet a) modified for a three-way factorial design with two levels of the amino acid mixture (61 and 122 mM), two levels of sucrose (10% and 20%), and three levels of sinigrin (Sigma) (0, 5, and 10 mM). These amino acid and sucrose concentrations were within the range of values recorded in the phloem sap of herbaceous plants (Barlow and Randolph, 1978; Fukumorita and Chino, 1982; Chino et al., 1986; Hayashi and Chino, 1986; Fisher and Gifford, 1986). Because no data on sinigrin concentrations in phloem sap were available, sinigrin concentrations were based on assays of tissue homogenates of black mustard (Feeny and Rosenberry, 1982). Sinigrin was the only glucosinolate in the present study to be detected by GLC in the honeydew of aphids feeding on black mustard (S. Merritt, unpublished data). This experiment was conducted using the University of Michigan clone.

I conducted a subsequent experiment to extend the range of amino acid concentrations present in the diet. This second experiment used the University of California clone because the University of Michigan clone was no longer available. The experiment was a one-way design with three levels of the amino acid mixture (92, 184, and 276 mM) and constant levels of sucrose (15%) and sinigrin (0%).

Diets were prepared according to Kunkel (1977), filtered (0.45 μm) into sterile plastic vials under a laminar-flow hood, and stored at $-60°C$ until needed. All diets were used within 14 days of mixing.

Diets were offered in Parafilm sachets consisting of about 0.2 ml of diet

sandwiched between two layers of stretched Parafilm (Mittler and Dadd, 1964). To minimize microbial contamination, sachets were prepared according to published procedures (Kunkel, 1977) with two modifications. First, sachets were prepared under a laminar-flow hood with diets dispensed from sterile glass pipets. Second, the Parafilm was not surface sterilized. Instead, only the paper-covered side of the Parafilm was allowed to contact the diet (i.e., form the inside of the sachet).

Aphids were reared in cages in an incubator (21°C, 16L:8D) for five days. Cages were made from 3-cm segments of acrylic tubing (3.81 cm OD, 3.18 cm ID) covered with a Parafilm sachet on the top and plastic cap on the bottom. Aphids fed upside down by piercing the Parafilm membrane and imbibing the liquid diet. Aphids were transferred to cages with fresh sachets every three days.

Each cage was started with five adult aphids that were allowed to feed and reproduce for five days, after which honeydew collection began. To collect honeydew, the bottom of each cage was removed, and the cage was nested in a 50-ml polypropylene beaker containing about 20 ml of technical grade (94%) 1-hexadecene (mp, 4°C; bp, 274°C) (Aldrich). Honeydew excreted by the aphids fell into the 1-hexadecene in the beaker and accumulated below the surface, which prevented honeydew evaporation so that solute concentrations could be measured. Honeydew from each cage was removed daily with a plastic micropipet tip, pooled with honeydew collected previously from the same cage, and stored under 1-hexadecene at −60°C until chemical analysis. About 0.1–0.2 ml of honeydew was recovered from each cage in six days of collection.

Honeydew Collection from Aphids Fed on Black Mustard. Black mustard plants were grown from seed (obtained from V & J Seed Farms, Woodstock, Illinois) under natural light from December to March in a greenhouse at the University of Michigan Matthaei Botanical Gardens. Plants were grown individually in 20.3-cm pots in potting soil (Fison's Sunshine Mix No. 4), watered daily, and fertilized every two weeks. After the plants had bolted, aphids were placed on leaves of four developmental stages on each plant: (1) young actively growing leaves; (2) mature leaves (dark green); (3) presenescent leaves (light green); and (4) senescent leaves (yellow-green to yellow). To collect honeydew, a plastic cup containing 1-hexadecene was positioned under each aphid-infested leaf. Each cup was supported by an acrylic ring that was attached to a ring stand by a steel rod. Honeydew from each leaf was collected as described above, pooled with honeydew collected previously from the same leaf, and stored under 1-hexadecene at −20°C until chemical analysis. About 0.2–0.3 ml of honeydew was recovered from each leaf in seven days of collection.

Chemical Analysis of Honeydew. The amino acid concentration in honeydew was measured colorimetrically (Coleman Junior Spectrophotometer, model 6C; or Sequoia-Turner, model 340) by the ninhydrin method (Spies, 1957). Concentrations were expressed in asparagine equivalents because asparagine was

the dominant amino acid in the artificial diets (Kunkel, 1977) and is often present in high concentration relative to other amino acids in the phloem sap of herbaceous plants (e.g., Pate et al., 1974; Barlow and Randolph, 1978; Fukumorita and Chino, 1982; Wink and Witte, 1984; Hayashi and Chino, 1986).

The sugar concentration in honeydew was measured colorimetrically (Coleman Junior Spectrophotometer, model 6C) by the phenol–sulfuric acid method (Hodge and Hofreiter, 1962). Sugar concentrations were expressed in sucrose equivalents because sucrose was the sugar in the artificial diets (Kunkel, 1977) and is the dominant carbohydrate in the phloem sap of herbaceous plants (e.g., Pate et al., 1974; Barlow and Randolph, 1978; Fukumorita and Chino, 1982; Hayashi and Chino, 1986), including crucifers (Pate, 1973).

Glucosinolate concentrations in honeydew were measured by gas chromatography (GC) of isothiocyanate hydrolysis products following the procedures of Rodman (1978) and Blau and Hanscom (1986). Phosphate buffer, ascorbate, and myrosinase were prepared according to Rodman (1978). Measured honeydew samples (0.025–0.125 ml) were placed into 1/2-dram shell vials along with phosphate buffer (0.450 ml), ascorbate solution (0.020 ml), myrosinase extract (0.010 ml), and methylene chloride (0.500 ml), which contained 0.01% *n*-undecane as an internal standard. Vials were capped and shaken gently at room temperature for 12 hr, after which the methylene chloride phase, without concentration, was subjected to analysis on a Hewlett-Packard 5890 gas chromatograph furnished with a 30-m column coated with a 100% methyl polysiloxane stationary phase. Both injector and FID temperatures were 250°C. Oven temperature was maintained at 90°C for 1 min and then increased at 50°C/min. Glucosinolates were identified and quantified by comparing retention times and peak areas with standards. Identifications were confirmed by GC-MS. Up to 75 samples per day were analyzed following this procedure.

Mean extraction efficiency of sinigrin standards was 80.6% (SD = 10.9%, $N = 35$). To avoid underestimating sinigrin concentrations in honeydew, estimates were based on a standard curve.

Statistical Analysis. All statistical analyses were done using SYSTAT (Wilkinson, 1990). Stepwise multiple linear regression was performed on various combinations of the independent variables (i.e., concentrations of chemicals in the honeydew), both transformed and untransformed, to generate equations for estimating concentrations of amino acids, sugar, and sinigrin in artificial diets from concentrations of these chemicals in aphid honeydew. These regressions are for predictive purposes only and are not meant to characterize mechanistic relationships. Ideally, a range of concentrations of diet components should be used to reliably characterize the relationship between aphid diets and honeydew. However, because the experiment was a factorial design, only two or three levels of diet components were feasible. As a result, some precision will be lost when estimating the concentrations of chemicals in phloem sap using these

equations. Furthermore, estimated concentration of sinigrin in young leaves was far higher than the highest diet concentration used to generate the equations; therefore, the highest diet concentration is presented as a conservative estimate of the concentration present, assuming a monotonically increasing relationship.

Estimated concentrations of amino acids in phloem sap were log transformed to obtain homogeneous variances, after which pairwise comparisons were made using a Tukey HSD test. Variances in the estimated concentrations of sugars in phloem sap could not be made homogeneous, so pairwise comparisons were made using Mann-Whitney U tests with alpha adjusted to 0.008 (0.05/6) for multiple comparisons.

Pairwise comparisons of means for estimated levels of sinigrin were tested using two-sample t tests when both values were within the range of diet concentrations used to generate the equations. When one of the values has higher than the highest diet concentration used, comparisons were done using one-sample t tests with the highest diet concentration (10 mM) used as the known mean. Estimated concentrations of sinigrin in the phloem sap of mature, presenescent, and senescing leaves were log $(X + 1)$ transformed to satisfy the assumptions of normality and homogeneity of variance. Alpha was adjusted to 0.008 (0.05/6) for multiple comparisons.

RESULTS

To estimate the composition of diets from the composition of honeydew, the following equations were generated relating the concentrations of amino acids (AA), sugar (SU), and sinigrin (SI) measured in the honeydew (h) of aphids fed on artificial diets (hAA, hSU, hSI, respectively) with the dietary (d) concentrations of these chemicals (dAA, dSU, dSI) (Table 1). In the parentheses below each equation is the range of concentrations in the diet component over which these equations apply and the proportion of the variance in the diet component explained by the equations (i.e., r^2).

$$dAA\ [\text{mM}] = 24.6 + 28.3\ (hAA\ [\text{mM}])^{1/2}$$

(range: 61–276 mM; $r^2 = 0.968$)

$$dSU\ [\%] = -4.05 + 4.59\ (hSU\ [\%])^{1/2} + 1.81\ (hAA\ [\text{mM}])^{1/2}$$

(range: 10–20%; $r^2 = 0.961$)

$$dSI\ [\text{mM}] = 0.438 + 0.405\ (hSI\ [\text{mM}])\ (hSU\ [\text{mM}])^{1/2}$$

(range: 0–10 mM; $r^2 = 0.940$)

TABLE 1. CONCENTRATIONS OF AMINO ACIDS, SUGARS, AND SINIGRIN IN HONEYDEW FROM APHIDS FED ON ARTIFICIAL DIETS

Diet concentration			Honeydew concentration (mean ± SEM)[a]		
Amino acids (mM)	Sucrose (%)	Sinigrin (mM)	Amino acids (mM)[b]	Sugars (%)[c]	Sinigrin (mM)
61	10	0	1.74 ± 0.062	6.38 ± 0.288	0.00 ± 0.000
		5	1.82 ± 0.046	6.58 ± 0.151	4.19 ± 0.141
		10	2.31 ± 0.072	7.23 ± 0.181	8.86 ± 0.533
	20	0	2.09 ± 0.062	21.10 ± 0.960	0.00 ± 0.000
		5	2.61 ± 0.059	19.47 ± 0.669	2.20 ± 0.068
		10	2.66 ± 0.090	22.08 ± 0.690	4.57 ± 0.215
92	15	0	6.24 ± 0.189	ND	ND
122	10	0	12.45 ± 0.485	3.01 ± 0.114	0.00 ± 0.000
		5	12.43 ± 0.337	2.78 ± 0.171	6.68 ± 0.240
		10	11.85 ± 0.427	2.83 ± 0.082	13.62 ± 0.587
	20	0	9.92 ± 0.223	15.62 ± 0.447	0.00 ± 0.000
		5	8.90 ± 0.169	15.95 ± 0.348	2.73 ± 0.129
		10	9.50 ± 0.225	16.78 ± 0.849	6.05 ± 0.338
184	15	0	24.56 ± 0.498	ND	ND
276	15	0	88.02 ± 2.656	ND	ND

[a] Sample size = 10 for all means. ND, not determined.
[b] Asparagine equivalents.
[c] Sucrose equivalents.

TABLE 2. CONCENTRATIONS OF AMINO ACIDS, SUGARS, AND SINIGRIN IN HONEYDEW FROM APHIDS FED ON LEAVES OF BLACK MUSTARD

	Honeydew concentration (mean ± SEM)			
Leaf age	Amino acids (mM)[a]	Sugars (%)[b]	Sinigrin (mM)	*N*
Young	56.1 ± 11.99	13.7 ± 0.77	15.41 ± 2.040	9
Mature	5.2 ± 1.52	18.9 ± 0.81	0.74 ± 0.131	10
Presenescent	3.7 ± 0.56	18.7 ± 0.69	0.34 ± 0.104	10
Senescent	52.6 ± 13.97	14.2 ± 1.54	2.17 ± 0.624	10

[a] Asparagine equivalents.
[b] Sucrose equivalents.

TABLE 3. ESTIMATED CONCENTRATIONS OF AMINO ACIDS, SUGARS, AND SINIGRIN IN PHLOEM SAP OF BLACK MUSTARD

Leaf age	Estimated Honeydew Concentration (mean and 95% CI)[a]			
	Amino acids (mM)[b]	Sugars (%)[c]	Sinigrin (mM)[d]	N
Young	215.7a (167.5–277.8)	25.8a (23.0–28.6)	>10.00a	9
Mature	83.4b (71.3–97.6)	19.8b (19.0–20.5)	1.66bc (1.13–2.32)	10
Presenescent	77.0b (69.1–85.9)	19.2b (18.4–20.0)	0.96c (0.58–1.43)	10
Senescing	198.5a (148.6–265.3)	25.1a (22.6–27.5)	3.02b (1.75–4.88)	10

[a] Means in a column followed by different letters differ at $P < 0.05$.
[b] Asparagine equivalents back-transformed from log (X).
[c] Sucrose equivalents.
[d] Back-transformed from log ($X + 1$). Estimates for sinigrin in young leaves were far higher than the highest diet concentration used to generate the equations; therefore, the highest diet concentration is presented as a conservative estimate of the concentration present, assuming a monotonically increasing relationship.

Applying these equations to the concentrations of chemicals measured in honeydew of aphids fed on black mustard (Table 2) gave estimates of the concentrations of chemicals in phloem sap (Table 3). Phloem sap in young and senescing leaves contained higher amino acid and sugar concentrations than phloem sap in mature and presenescent leaves. A high concentration of sinigrin occurred only in phloem sap in young leaves. Although the concentration of sinigrin was slightly higher in phloem sap in senescing leaves than in mature and presenescent leaves, sinigrin concentrations in these leaves were far lower than those in young leaves.

Based on the nutrient and allelochemical profile of their phloem sap, leaves of black mustard fell into three classes: (1) young leaves with high concentrations of nutrients and sinigrin, (2) mature and presenescent leaves with low concentrations of nutrients and sinigrin, and (3) sensecing leaves with high concentrations of nutrients and low concentrations of sinigrin.

DISCUSSION

The conclusions of this study rest on the reliability of the estimate of phloem sap composition. Two lines of evidence suggest that the estimates are reasonable. First, the artificial diet experiment shows that diet composition can

be accurately estimated from honeydew composition. Indeed, the regressions explained 94% or more of the variance in diet concentrations of amino acids, sucrose, and sinigrin. Second, the estimates in the present study compare favorably with values obtained in other studies. The estimated concentrations of amino acids in the phloem sap of black mustard (77–216 mM) (Table 3) are similar to those measured in the phloem sap of a variety of herbaceous plant species (112–1600 mM) (Barlow and Randolph, 1978; Fukumorita and Chino, 1982; Pate et al., 1984; Fisher and Gifford, 1986; Weibull, 1988). Similarly, estimated sugar concentrations (19.1–25.8%) (Table 3) fall within the range recorded in herbaceous plants (4.3–30.8%) (e.g., Pate, 1973; Pate et al., 1974; Fukumorita and Chino, 1982; Fisher and Gifford, 1986; Ohshima et al., 1990). However, feeding by aphids has been shown to enhance the translocation of sugars into infested leaves, and thus it is possible that these estimates are high (Way and Cammell, 1970).

Nevertheless, there remains some concern about the accuracy of the estimates of phloem sap composition both because the equations are based on very few levels of diet components and because some estimates fall far outside the range of concentrations over which the equations apply. In addition, the estimation procedure is sensitive to the presence of nontarget compounds that give positive reactions with the assays. This latter consideration is unlikely to be important for sinigrin because of the high specificity of the GC test for this chemical. However, the ninhydrin and phenol–sulfuric acid assays are not as specific, and it is known that phloem sap contains a number of chemicals that give positive reactions with each (e.g., nonprotein amino acids, nucleotides for ninhydrin assay; flavonoids, phenolics, complex carbohydrates for phenol–sulfuric assay). The estimation procedure assumes the presence of insignificant amounts of these interfering chemicals, and estimates will be inflated depending on the levels of these chemicals in the phloem sap and their metabolism in the gut of aphids.

It is generally believed that phloem sap contains only low concentrations of allelochemicals (Mullin, 1986; Dreyer and Campbell, 1987; Klingauf, 1987a; Brattsten, 1988). However, the estimates in this study of the concentrations of sinigrin in phloem sap in young leaves (>10.00 mM, i.e., >0.416% fresh weight) are as high as those measured in foliage of black mustard (0.165–0.632% fresh weight) (Feeny and Rosenberry, 1982). Indeed, the sinigrin concentration in phloem sap in young leaves might be even higher because the highest diet concentration is presented as a conservative estimate of the concentration present. Thus, the present study adds to the growing body of evidence documenting high concentrations of allelochemicals in phloem sap (Wink et al., 1982; Wink and Witte, 1984) and in related vascular exudates (Treutter et al., 1985; Niemeyer, 1988; Peng and Miles, 1991).

The assumption that allelochemicals occur at low concentrations in phloem

sap has led many authors to conclude that phloem-borne allelochemicals do not act as defenses against sap-feeders (Mullin, 1986; Dreyer and Campbell, 1987; Klingauf, 1987a; Brattsten, 1988). However, the presence of high allelochemical concentrations indicates that this conclusion needs to be reconsidered. Furthermore, the occurrence of within-plant variability in allelochemical concentrations in phloem sap suggests that some plant parts may be better defended than others. For example, the high sinigrin concentration in young leaves implies that they will be better defended against nonadapted sap-feeding herbivores than will mature, presenescent, or senescent leaves. Similar within-plant variability was also documented in a species of lupine, *Lupinus consentinii*, in which it was found that phloem sap from fruits contained over three times the alkaloid concentration as phloem sap in stems (Wink et al., 1982). These data support the hypothesis that young leaves should be better defended against herbivores because they have higher relative fitness value to the plant than do mature, presenescent, and senescing leaves (McKey, 1979; Rhoades, 1979).

Nutrient concentrations in phloem sap also varied within plants. Young and senescent leaves of black mustard contained higher amino nitrogen and sugar than did mature and presenescent leaves (Table 3). Similar patterns have been reported for nitrogen in phloem sap. Phloem sap in young and senescing foliage of *Salix acutifolia* contains about three times the nitrogen as phloem sap of mature foliage (Mittler, 1958). Likewise, phloem exudate from growing tissue (stem tops, developing fruits) of *Lupinus albus* contains about 1.7 times the amino nitrogen as exudate from mature leaves (Pate et al., 1979). Moreover, as mature leaves senesce, amino nitrogen content increases 1.7 times (Pate et al. 1979). This pattern of high amino nitrogen concentration in phloem in senescing leaves probably arises from the breakdown of leaf proteins and subsequent translocation back to the plant of the resulting amino acid components prior to leaf abscission (Stoddart and Thomas, 1982). Pate et al. (1979) also examined within-plant variation in sucrose concentrations. In contrast to the present study, concentrations in exudate from growing tissue of *Lupinus albus* were lower (0.7–0.8 times) than in exudate from mature leaves.

The within-plant variability in phloem sap composition documented here may affect the performance and feeding preferences of sap-feeding herbivores. Numerous studies have shown that variation in dietary concentrations of amino acids and sucrose affects aphid growth, survival, and reproduction (reviewed in Klingauf, 1987a). Allelochemicals too can alter food quality for aphids, acting as arrestants, deterrents, or phagostimulants (see references in Klingauf, 1987b; Risebrow and Dixon, 1987; Montllor, 1991). In plants, variability in these chemicals will make some feeding sites more suitable than others. For example, by feeding on senescing leaves of black mustard, a phloem-feeding insect would simultaneously maximize its nutrient intake and minimize its exposure to sinigrin. This explanation appears to account for the occurrence of highest densities

of the green peach aphid on senescing leaves of crucifers (van Emden et al. 1969). Within-plant variation in phloem chemistry may similarly explain some of the patterns of host use seen in other phloem-feeding insects (Dixon, 1976; Wink et al. 1982; Whitham, 1983; Wink and Römer, 1986).

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INFLUENCE OF ALKALOID CONCENTRATION ON ACCEPTABILITY OF TALL LARKSPUR (*Delphinium* SPP.) TO CATTLE AND SHEEP

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Abstract—Tall larkspur (*Delphinium* spp.) is a serious toxic plant problem on western U.S. ranges. The major toxins in tall larkspur are methyllycaconitine (MLA) and 14-deacetylnudicauline (14-DAN); the sum of both is termed the toxic alkaloid concentration. Toxic alkaloids comprise about 20–50% of the total alkaloid concentration in tall larkspur. Toxic and total alkaloid concentration generally declines with maturity, whereas cattle and sheep consumption of larkspur typically increases with plant maturity. We hypothesized that cattle and sheep consumption of tall larkspur was negatively related to higher concentrations of total or toxic alkaloid. We compared consumption of several collections of dried, ground larkspur and fresh larkspur in a series of trials. In another trial, a crude alkaloid fraction was extracted with ethanol, added to alfalfa hay, and consumption compared to untreated alfalfa hay, alcohol-treated hay, and the essentially alkaloid-free plant residue. In all cases we correlated amounts eaten with total and toxic alkaloid concentration. A grazing trial was also conducted to relate larkspur consumption over time to alkaloid concentrations. Total alkaloid concentrations in dried, whole-plant collections ranged from 9.3 to 38.8 mg/g of dry weight, whereas toxic alkaloid concentrations varied from 0.0 to 7.1 mg/g. In one pen trial, cattle preferred a larkspur collection ($P < 0.01$) that contained no toxic alkaloids but had a high total alkaloid concentration (39 mg/g). There was no correlation ($P > 0.05$), however, between concentrations of total or toxic alkaloids and amount of dry plant consumed in this or any other trial. Conversely, sheep consumption tended to be negatively influenced by total and toxic alkaloid concentration ($P \leq 0.08$). In the trials with extract, cattle preferred the alcohol-treated hay and rejected the alkaloid-free residue ($P < 0.01$), whereas the alkaloid-

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treated hay was of intermediate acceptability. Cattle preferred the alkaloid-treated hay over the alkaloid-free residue, indicating that alkaloids did not deter consumption. Conversely, the alkaloid-treated hay was less preferred than either untreated or alcohol-treated hay, suggesting a negative effect on acceptability. There was no correlation between alkaloid concentration and amount of treated feed eaten. In field trials, the amount of composited, fresh leaves or flowers eaten by cattle was influenced by plant part ($P = 0.04$), but was not related ($P > 0.05$) to alkaloid concentration. Cattle preferred leaves over flowers when offered individual plants differing in phenological stage and/or amount of shade, but alkaloid concentration was not related to consumption. We conclude that knowledge of the concentration of total or toxic alkaloid in tall larkspur will give little or no indication of plant acceptability to cattle. Even though accurate predictions can be made about the potential toxicity of larkspur based on the concentration of toxic alkaloids, predictions about consumption must be based primarily on plant phenology.

Key Words—Plant toxins, palatability, diterpenoid alkaloids, methyllycaconitine, *Delphinium* spp.

INTRODUCTION

Tall larkspur (*Delphinium* spp.) is a serious toxic plant problem on western U.S. ranges. Cattle grazing summer ranges sometimes consume large quantities (>30% of diets) of tall larkspur (Pfister et al., 1988a,b) and are often fatally poisoned as a result. Death losses average 5% and may exceed 15% each year on grazing allotments where larkspur is prevalent.

The major toxins in tall larkspur are the diterpenoid alkaloids methyllycaconitine (MLA) and 14-deacetylnudicauline (14-DAN) (Manner et al., 1993). Collectively, these two alkaloids account for about 90% of the toxicity of tall larkspur (Manners and Panter, unpublished data, 1995). These toxins block acetylcholine receptors in the central and peripheral nervous systems, resulting in muscular paralysis and eventually death (Dobelis et al., 1993). Tall larkspur contains other diterpenoid alkaloids in varying quantities (e.g., deltaline), but these other alkaloids are relatively nontoxic (Manners et al., 1993). Thus, in examining the alkaloid concentration of MLA and 14-DAN, the sum of these two toxic alkaloids (MLA + 14-DAN) are termed the toxic alkaloid concentration, and the combined concentration of all alkaloids are the total alkaloids.

The toxic and total alkaloid concentration in leaves generally declines as plants mature (Ralphs et al. 1988; Pfister et al., 1994a), whereas cattle and sheep consumption usually increases with plant maturity (Pfister et al., 1988a,b; Pfister and Manners, 1991, 1995; Ralphs et al., 1991; Ralphs and Olsen, 1992). Based on this inverse relationship, we hypothesized that toxic or total alkaloid concentration negatively influences the acceptability of tall larkspur to cattle and sheep. Our objective in this study was to test this hypothesis using both dried and fresh tall larkspur plant in a series of pen and field trials.

METHODS AND MATERIALS

Cattle Trials

Dried Plant Pen Trials (Trials 1 and 2). Entire tall larkspur plants (*Delphinium barbeyi* and *D. occidentale*) were harvested at various phenological stages (Table 1), air-dried at 25°C, ground to pass a 2-mm screen in a Wiley mill, and stored in sealed plastic bags at 16°C until use. Larkspur alkaloids are stable indefinitely under these conditions. In trial 1, we used ground larkspur collected during 1989, whereas in trial 2 we used ground larkspur collected over a four-year period. We ground the larkspur in order to offer homogenous plant material to subjects and to ensure that subjects could not select for specific plant parts. In both trials, larkspur material was collected at different stages of maturity and/or locations in order to maximize potential differences in alkaloid concentration, since immature larkspur generally contains the highest concentration of total and toxic alkaloids. Ground plant material was subsampled for alkaloid analysis in trials 1 and 2. We hypothesized that immature larkspur would be less acceptable to cattle than mature plant because of a higher alkaloid concentration.

Four yearling Hereford × Angus heifers (290 kg) were used during the spring of 1992 to evaluate the acceptability of the ground larkspur. Animals were group fed ground (2-mm screen) alfalfa hay for 21 days to habituate them to ground feed. Cattle were individually trained in the test procedure for four days. Each subject was given several opportunities to eat small amounts (about 100 g) of rolled barley, and later ground alfalfa, from four feed pans evenly spaced along the fence on one side of the pen. The 36- × 18- × 15-cm (length × width × depth) test pans were placed in specially constructed frames that hooked into the fence in the animal's pen. Animals had free access to all pans for 5 min. The subjects quickly learned to move from pan to pan, eating the small amount of feed in each pan. During testing, animals were fasted until mid-morning when they were tested; they were fed their basal ration of alfalfa hay at midday.

We determined in pilot tests that cattle could consume about 300–500 g of ground alfalfa hay during 5-min test periods. In the test procedure, about 500 g of larkspur material was weighed into each pan, and after an overnight fast, individual animals were offered all four choices simultaneously in the pans for 5 min. Each day the larkspur collections were allocated to a different position in the pen, and each test was run for either three or four consecutive days. Cattle must ingest 8–11 mg of toxic alkaloids per kilogram body weight within a couple of hours for subclinical toxicosis to occur (Pfister et al., 1994b), and animals did not eat enough larkspur to approach this toxic threshold.

Multiple 4 × 4 Latin squares were used initially to assess preference for four different larkspur collections (Borman et al., 1991). Each animal in a pen

TABLE 1. LARKSPUR SPECIES OR FEED TYPE, PHENOLOGICAL STAGE, HARVEST DATE, LOCATION OF PLANT COLLECTION, AND PHYSICAL FORM OFFERED TO CATTLE OR SHEEP DURING ACCEPTABILITY TRIALS.

Species or feed	Phenological stage	Harvest date	Location	Form offered
Trial 1: Cattle and sheep				
Delphinium occidentale	vegetative	6/21/1989	Oakley, ID	air-dried, ground
Delphinium occidentale	flower	7/18/1989	Oakley, ID	air-dried, ground
Delphinium barbeyi	vegetative	7/1/1989	Manti, UT	air-dried, ground
Delphinium barbeyi	flower	8/15/1989	Manti, UT	air-dried, ground
Trial 2				
Delphinium barbeyi	pod	8/25/1986	Manti, UT	air-dried, ground
Delphinium barbeyi	flower	8/12/1987	Manti, UT	air-dried, ground
Delphinium barbeyi	vegetative	7/6/1989	Manti, UT	air-dried, ground
Delphinium barbeyi	flower	8/5/1990	Manti, UT	air-dried, ground
Trial 3				
D. barbeyi extracted residue[a]	flower	7/93	Manti, UT	air-dried, ground
D. barbeyi crude extract[b]	flower	7/93	Manti, UT	mixed with ground hay
Alfalfa + EtOH[c]				air-dried, ground hay
Alfalfa untreated				air-dried, ground hay

Trial 4				
Delphinium barbeyi	full flower[d]	7/16/92	Yampa, CO	fresh, composited flowers
Delphinium barbeyi	early flower[e]	7/16/92	Yampa, CO	fresh, composited leaves
Delphinium barbeyi	early flower	7/16/92	Yampa, CO	fresh, composited flowers
Delphinium barbeyi	full flower	7/16/92	Yampa, CO	fresh, composited leaves
Trial 5				
D. barbeyi plant 1	full flower	7/17/92	Yampa, CO	fresh, cut stalks
D. barbeyi plant 2	early flower	7/17/92	Yampa, CO	fresh, cut stalks
D. barbeyi plant 3	full flower	7/17/92	Yampa, CO	fresh, cut stalks
D. barbeyi plant 4	early flower	7/17/92	Yampa, CO	fresh, cut stalks
Trial 6				
D. barbeyi plant 1	full flower (shaded)	8/7/92	Yampa, CO	fresh, cut stalks
D. barbeyi plant 2	full flower (shaded)	8/7/92	Yampa, CO	fresh, cut stalks
D. barbeyi plant 3	full flower (no shade)	8/7/92	Yampa, CO	fresh, cut stalks
D. barbeyi plant 4	full flower (no shade)	8/7/92	Yampa, CO	fresh, cut stalks
Trial 7				
D. barbeyi plant 1	full pod[f] (no shade)	8/19/92	Yampa, CO	fresh, cut stalks
D. barbeyi plant 2	full pod (shaded)	8/19/92	Yampa, CO	fresh, cut stalks
D. barbeyi plant 3	early pod[g] (shaded)	8/19/92	Yampa, CO	fresh, cut stalks
D. barbeyi plant 4	full pod (no shade)	8/19/92	Yampa, CO	fresh, cut stalks

[a] Larkspur plant residue was the material remaining after extraction with ethanol
[b] Crude extract was extracted, tar-like material; the extract was mixed with ground alfalfa hay
[c] Ethanol was mixed with ground alfalfa hay, then hay was air-dried.
[d] Flowering racemes fully elongated; sepals fully opened and conspicuous
[e] Flowering racemes fully elongated; sepals closed- not visible or inconspicuous
[f] Pods fully developed
[g] Pods partially formed; some flower parts visible

constituted a complete Latin square, with factors: animal (i.e., square), four positions within the pen, four days, and four different larkspur collections (Table 1). After the initial 4 × 4 Latin square was completed for trial 1, the plant material with the highest ranking (i.e., most acceptable) was eliminated, and the test was repeated using three randomly selected animals in a 3 × 3 Latin square; this elimination and retesting procedure was not done during trial 2. We retested to determine if acceptability of the remaining choices was consistent with previous trials.

Larkspur Extract Trial (Trial 3). This trial with ground plant tissue was conducted to assess the acceptability of tall larkspur with the alkaloid fraction removed and to determine if this extracted crude fraction was acceptable when added to alfalfa hay. We hypothesized that cattle would accept the alkaloid-free tall larkspur residue and that cattle would not accept the alfalfa hay when mixed with the crude alkaloid-rich extract. Tall larkspur (*D. barbeyi*) plants were collected during August 1993 near Manti, Utah, on the Wasatch Plateau at about 3000 m elevation, dried at 20°C, and ground to pass a 2-mm screen. Plant material (3.3 kg air-dry weight) was exhaustively extracted with 95% ethanol (EtOH) for four days using a large Soxhlet apparatus to remove all alkaloid compounds. The solvent was removed from the crude alkaloid extract in a rotary evaporator. The tarlike crude alkaloid extract was reconstituted with saline at 3.25 ml/g of extract. The crude extract was evenly applied to 3.3 kg of ground (2-mm screen) alfalfa hay, and air-dried at 20°C for four days. This trial had two controls: untreated, ground (2-mm screen) alfalfa hay and ground alfalfa hay treated with 95% EtOH at 0.75 ml/g of hay. The EtOH was evenly mixed with the hay until all the hay was moist, then the EtOH hay was allowed to dry for four days at 20°C. The extracted larkspur plant residue and crude plant extract containing larkspur alkaloids were subsampled for alkaloid analysis.

The same testing protocol was followed in this trial as in the preceding cattle trials. The four test animals were 3-year-old cows (Hereford × Angus cross; 470 kg); animals were not naive to larkspur, and they were habituated to the procedure before testing.

Fresh Plant Trials (Trials 4–7). Because dried, ground larkspur is not the physical form encountered by grazing cattle, we also conducted acceptability trials using fresh plant material. As with the previous trials, we harvested tall larkspur plants at different phenological stages and/or grown in full or partial sunlight; the intent in all plant collections was to simultaneously offer plants with potential differences in total and total toxic alkaloid concentrations. It was not possible to determine the alkaloid concentration of fresh plants before trials began; hence, we selected plants based on two assumptions: (1) plants grown in full sunlight have higher concentrations of total alkaloids compared to larkspur grown in partial shade (Pfister et al., 1988a), and (2) toxic and total alkaloid concentrations will be lower in mature compared to less mature plants. We

hypothesized that leaves or flowers from mature larkspur, with lower alkaloid concentrations, would be more acceptable to cattle compared to immature plant material; and, furthermore, that larkspur at the same growth stage grown in partial shade, with presumably lower alkaloid concentrations, would be more acceptable to cattle compared to larkspur grown in full sunlight.

Trials with freshly harvested tall larkspur (*D. barbeyi*) were conducted during July and August 1992 near Yampa, Colorado, using composite plant collections (trial 4, Table 1) or individual plants (trials 5–7, Table 1). The design for trials 4–7 was a 4 × 4 Latin square with each animal comprising a separate square.

Four yearling Hereford × Angus heifers (320 kg) were used as test animals. A portable corral was constructed adjacent to a larkspur-infested site at 2866 m elevation and a 5- × 8-m pen used for these trials. Animals were gentle and habituated to the test procedure before testing began. Testing was done individually in the morning, with cattle grazing during the remainder of the day.

Composite collections of larkspur leaves and flowers were tested as fresh material (trial 4, Table 1). Numerous (i.e., > 15) plants in the same phenological stage were stripped of leaves and flowers on the morning of the first test. Sufficient material was collected to last for all tests. The material was offered in feed pans in different pen locations as noted above. All four choices were offered simultaneously in this and subsequent trials. Between tests on the subsequent three days, the composited collections were sprinkled with water and stored in plastic bags at 10°C. On the last test day the material was not visibly wilted.

In Trials 5–7, individual larkspur plants were cut at ground level and preserved for the remainder of the trials (Table 1). Multiple stalks from the same plant were bound into a single sheaf near the bottom using cord. Plants were offered to cattle after securing them to the pen fence. When not being offered to cattle, individual plants were kept fresh and turgid by placing them under a shaded canopy with the bottom portion of each plant immersed in water. At this high mountain elevation, daytime temperatures are sufficiently cool that wilting did not occur. Each day individual animals were released into a test pen and allowed 5-min access to larkspur. The number of bites by plant part (leaf, stem, or reproductive parts) from each plant was recorded by a single observer. Each day plants were rotated to a new position.

Subsamples (10 g) of fresh flowers and leaves in trial 4 were frozen, freeze-dried, and ground through a 2-mm screen. Alkaloid concentration was determined for individual plants in trials 5, 6, and 7 by harvesting leaves and reproductive parts (i.e., flowers or pods) from two randomly selected stems immediately after the plant was cut; these samples were immediately frozen, then later freeze-dried and ground (2-mm screen) for analysis. The alkaloid concentration of leaves harvested from one or more stems is representative of leaves from the whole-plant (Manners and Pfister, unpublished data).

Lamb Trial

Sheep are not often intoxicated by tall larkspur because they are much less susceptible to the toxic alkaloids. Sheep are sometimes used to graze a pasture before cattle to reduce subsequent larkspur availability to cattle by trampling or consumption (Ralphs et al., 1991). Grazing trials with sheep have shown that sheep are reluctant to eat immature tall larkspur, but will eat mature larkspur (Ralphs et al., 1991, Ralphs and Olsen, 1992); thus, we hypothesized that alkaloid concentration was inversely related to acceptability to sheep. To test this hypothesis, four 6-month-old weaned male lambs were offered ground *D. barbeyi* or *D. occidentale* collected in the vegetative or flower stage (Table 1). We used the same material in this trial as in cattle trial 1. Sheep were naive to larkspur. Lambs were habituated to consuming 1.5 kg/day of ground alfalfa for 21 days. As in the cattle trials (described above), sheep were tested first in a 4 × 4 Latin square, then tested in a 3 × 3 Latin square trial with the most acceptable feed removed. At the end of the 3 × 3 trial, we removed the most acceptable feed, and conducted another trial using a 2 × 2 crossover design with four sheep, two feeds, and two positions over two days.

Cattle Field Grazing Trial

Grazing cattle select leaves from some tall larkspur plants and avoid others; furthermore during some years cattle select large amounts of tall larkspur, whereas during other years cattle may totally ignore larspur in the same location (Pfister et al., 1988a,b; Pfister and Manners, 1991, 1995). We hypothesized that these selection patterns are at least partially mediated by alkaloid concentrations; we predicted that highly selected plant samples would have lower alkaloid concentrations than plants in the general population. We also predicted that consumption of tall larkspur by grazing cattle would show an inverse relationship to total or toxic alkaloid concentration.

Six 1-year-old heifers (302 kg) were grazed from July 15 to August 26, 1992, on a tall larkspur-infested range at 2866 m elevation near Yampa, Colorado. The 8-ha pasture was enclosed with electric fence, and the cattle were allowed to freely graze from 07:00 to 19:00 hr each day. Cattle were not naive to tall larkspur, as they had grazed on similar tall larkspur-infested pastures the previous summer. Tall larkspur was in the early flower stage (i.e., reproductive racemes elongated, but flowers not open) when the trial began. At the end of the trial, all tall larkspur plants were in the pod stage, and some plants were senescing.

Cattle diets were determined using a bite-count procedure (Pfister et al., 1988a,b) on the seven dates that plant samples were collected for alkaloid analysis. Individual subjects were observed for 5-min periods in a predetermined order during all daylight hours when cattle were actively grazing; at the end of

the 5-min observation period the observer would observe the next animal in the sequence. We recorded bites of tall larkspur plant parts (flower, pod, or leaf), grasses, other forbs, shrubs, and monkshood (*Aconitum columbianum*). We defined an individual bite as a single cropping motion, always indicated by a head jerk, often acompanied by a visible sweep of the tongue, independent of chewing motions.

Tall larkspur leaves and reproductive parts from >20 plants were collected on seven dates to determine alkaloid concentration as larkspur matured. In addition, on three other occasions cattle were observed eating unusually large amounts of tall larkspur (i.e., >50% of diet for >1 hr), and remaining leaves from several partially consumed plants were collected and designated "palatable larkspur samples." Plant samples were composited, frozen at −20°C, freeze-dried, and ground to pass a 2-mm screen.

Plant Alkaloid Analysis

HPLC Toxic Alkaloid Analysis. One gram of ground, air-dried plant material was extracted in a Soxhlet apparatus with 80% EtOH. Further details of the HPLC procedure are given in Manners and Pfister (1993). Norditerpenoid alkaloids were detected by UV absorbance at 200 and 280 nm.

Total Alkaloid Analysis. Plant samples (100 mg) were weighed into a test tube and extracted with a mixture of $CHCl_3$ (4 ml) and 1 N HCl (4 ml) by mechanical rotation for 45 min. After centrifugation, the aqueous extract was removed and saved, and the plant material was extracted a second time with 1 N HCl (2 ml) for 5 min. The two aqueous extracts were combined and made alkaline with concentrated NH_4OH to pH 9. The samples were then extracted with $CHCl_3$ (2×), and the $CHCl_3$ solution was separated and filtered through Na_2SO_4. The solvent was removed by evaporation at 70°C under a stream of nitrogen. Carbon disulfide (2.0 ml) was added and the samples analyzed by FT-IR spectroscopy for total alkaloids using a quantitative partial least-squares model based on gravimetric analyses of the identically prepared total alkaloid fraction from a set of standard samples (D. Gardner, unpublished data, 1995).

Statistical Analysis

The statistical model for the Latin square trials was: intake = animal in pen (i.e., squares), position in pen, day in pen, larkspur collection or plant (i.e., treatment), error. The protected LSD procedure was used to compare treatment means after a significant F test ($P < 0.05$). Although there were confounding factors (e.g., years and phenology in trial 2; amount of shade in trials 6 and 7), we consider this to be trivial in the analysis because the independent variable of interest was alkaloid concentration. We determined Pearson correlation coefficients (r) relating alkaloid concentrations to: (1) amount eaten (grams) in trials

1–3 (dried, ground material); (2) amount eaten (grams) in trial 4 (composite fresh material); and (3) total bites of leaves, flowers, and pods from individual plants in trials 5–7, with these three trials grouped together for the correlation analysis.

Bite count data from the grazing trial were transformed using an arcsin-square root transformation. The grazing trial was analyzed using ANOVA procedures, with individual animals as blocks and sampling dates as treatments. The GLM procedure of SAS (1987) was used for data analysis. No statistical analysis was possible on corresponding alkaloid concentrations for each date, as composite samples were used and no measure of the variance was available.

RESULTS

Cattle Pen Trials

No differences in consumption due to plant collection were found in trial 1 ($P = 0.13$), although cattle did consume numerically more of the late season *D. barbeyi* and less of the early-season *D. barbeyi* (Table 2). Thus, we rejected the hypothesis that cattle would consume significantly more of the mature larkspur with a lower alkaloid concentration. When the late-season *D. barbeyi* was removed as a choice and the test rerun as a 3 × 3 Latin square, no differences in consumption were noted ($P = 0.79$) (Table 2). There was no relationship ($P > 0.05$) between total or toxic alkaloid concentrations and the amount of plant eaten (Table 3). There were significant correlations among the specific alkaloid concentrations, with the exception of 14-DAN.

In trial 2, cattle preferred the 1986 *D. barbeyi* collection ($P < 0.01$) over the other plant collections (Table 4). This collection was unusual in that it had no detectable toxic alkaloid concentration, but it did have a high concentration of total alkaloid. Thus, we rejected the hypothesis that total alkaloid concentration negatively influences consumption, but this trial suggested that toxic alkaloid concentration may influence acceptability. There was no correlation ($P > 0.05$), however, between concentrations of total or toxic alkaloids and amount of dry plant consumed in trial 2 (Table 5).

Cattle preferred both EtOH-treated hay and untreated hay during trial 3 (Table 4). The alkaloid-treated hay was of intermediate acceptability, whereas the alkaloid-free residue was rejected. During the 3 × 3 trial, when the EtOH-treated hay was withdrawn, the ranking remained the same, with cattle preferring untreated alfalfa, consuming moderate amounts of the alkaloid-treated hay, and rejecting the residue (Table 4). We rejected the hypothesis that cattle would preferentially accept the alkaloid-free residue over the alfalfa hay treated with the crude alkaloid extract. We could not reject the hypothesis of alkaloid influences because the alkaloid-treated hay was less preferred than either control

TABLE 2. TOTAL CONSUMPTION DURING TRIAL 1 OF DIFFERENT LARKSPUR COLLECTIONS (WHOLE PLANT), OFFERED AS AIR-DRIED, GROUND MATERIAL TO CATTLE AND SHEEP, AND CONCENTRATION OF METHYLLYCACONITINE (MLA), 14-DEACETYLNUDICAULINE (14-DAN), TOXIC ALKALOID, AND TOTAL ALKALOID IN PLANT MATERIAL

Trial/item	Phenological stage[a]	Consumption (g)[b]	$P > F$[c]	$LSD_{0.05}$	Concentration (mg/g dry wt)			
					MLA	14-DAN	Toxic	Total
Trial 1: 4 × 4, cattle			0.13					
D. occidentale	vegetative	609			6.9	0.2	7.1	35.9
D. occidentale	flower	583			2.3	0.05	2.4	12.2
D. barbeyi	vegetative	367			5.0	0.4	5.4	28.1
D. barbeyi	flower	842			1.2	0.4	1.6	10.2
Trial 1: 3 × 3, cattle			0.79					
D. occidentale	vegetative	545						
D. occidentale	flower	475						
D. barbeyi	vegetative	351						
Trial 1: 4 × 4, lambs			0.01	24.2				
D. occidentale	vegetative	158						
D. occidentale	flower	167						
D. barbeyi	vegatative	136						
D. barbeyi	flower	1048						
Trial 1: 3 × 3, lambs			0.05	34.7				
D. occidentale	vegetative	92						
D. occidentale	flower	294						
D. barbeyi	vegetative	107						
Trial 1: 2 × 2, lambs			0.61					
D. occidentale	vegetative	158						
D. barbeyi	vegetative	109						

[a]Stage of growth when larkspur was collected.
[b]Total consumption by cattle or sheep during Latin square trials.
[c]Probability of a greater F value for a feed (i.e., treatment) effect.

TABLE 3. PEARSON CORRELATION COEFFICIENTS (r) AND PROBABILITIES ($P > |r|$) FOR RELATIONSHIPS AMONG CONCENTRATIONS (mg/g DRY WEIGHT) OF TOTAL ALKALOID TOXIC ALKALOID, METHYLLYCACONITINE (MLA), AND 14-DEACETYLNUDICAULINE (14-DAN), AND AMOUNT OF LARKSPUR EATEN (g) IN TRIAL 1 BY CATTLE ($N = 7$) OR SHEEP ($N = 9$) USING DRIED, GROUND LARKSPUR SAMPLES.

	Total		Toxic		MLA		14-DAN	
	r	$P > r$	r	$P > r$	r	$P > r$	r	$P > r$
Cattle								
Eaten (g)	−0.54	0.45	−0.40	0.36	−0.40	0.37	−0.05	0.91
14-DAN	0.07	0.93	0.21	0.65	0.14	0.77		
MLA	0.99	0.006	0.99	0.0001				
Toxic	0.99	0.0008						
Sheep								
Eaten (g)	−0.62	0.08	−0.64	0.06	−0.66	0.05	0.23	0.55
14-DAN	0.21	0.59	0.18	0.64	0.10	0.80		
MLA	0.99	0.0001	0.99	0.0001				
Toxic	0.99	0.0001						

food. There was, however, no correlation ($P > 0.05$) between any alkaloid concentration and amount consumed (Table 5), but there were significant relationships among all the alkaloid components.

When fresh leaves and flowers were offered to cattle during trial 4, cattle rejected flowers and ate mostly leaves from plants in the full-flower stage ($P = 0.04$) (Table 4). The MLA concentration of the various composite collections varied greatly, but the range of total alkaloid concentrations was relatively small among the four collections. There was a biologically noteworthy, but statistically insignificant, negative relationship between amount of fresh plant eaten and total alkaloid concentration ($r = -0.74$, $P = 0.26$) (Table 5). Unlike the other trials, the only significant correlation among alkaloid concentrations in this trial was between MLA and toxic alkaloids (Table 5).

There were no differences ($P > 0.1$) in acceptability of individual plants in trials 5, 6, or 7 (Table 6). In trials 5 and 6, cattle ate no flowers, but cattle did take some bites of pods during trial 7 (Table 6). There was no correlation between alkaloid concentration and bites taken of plant material (Table 7). In general, all alkaloidal components were significantly correlated with one another. Taking trials 5–7 together, we rejected the hypothesis that mature larkspur would be more acceptable than less mature plant because of lower alkaloid concentrations. We were also unable to detect any significant impact from shade or full sun on amount of leaf material eaten by cattle.

TABLE 4. TOTAL CONSUMPTION BY CATTLE DURING TRIALS 2–4 OF DIFFERENT LARKSPUR COLLECTIONS OR TREATMENTS AND CONCENTRATION OF METHYLLYCACONITINE (MLA), 14-DEACETYLNUDICAULINE (14-DAN), TOXIC ALKALOID, AND TOTAL ALKALOID IN PLANT MATERIAL

Trial/item	Phenological stage[a]	Consumption (g)[b]	$P > F$[c]	$LSD_{0.05}$	Concentration (mg/g dry wt)			
					MLA	14-DAN	Toxic	Total
Trial 2: 4 × 4, Cattle[d]			0.01	73.1				
D. barbeyi	pod, 1986	1234			0.0	0.0	0.0	38.8
D. barbeyi	flower, 1987	355			2.3	0.1	2.4	9.3
D. barbeyi	vegetative, 1989	499			5.8	0.7	6.6	28.2
D. barbeyi	flower, 1990	316			2.7	0.2	3.0	16.4
Trial 3: 4 × 4, Cattle[e]			0.01	66.7				
Alfalfa		1813			0.0	0.0	0.0	0.0
Alfalfa + EtOH		1854			0.0	0.0	0.0	0.0
Larkspur extract + alfalfa		887			1.4	0.2	1.6	11.4
Larkspur plant residue		43			0.0	0.0	0.0	1.1
Trial 3: 3 × 3, cattle			0.01	85.9				
Alfalfa		1189						
Larspur extract + alfalfa		796						
Larkspur plant residue		64						
Trial 4: 4 × 4, cattle[f]			0.04	46.1				
D. barbeyi	leaves–early flower	260			4.7	0.2	4.9	7.4
D. barbeyi	flowers–early flower	20			1.9	0.09	2.0	6.9
D. barbeyi	leaves–full flower	455			2.6	0.09	2.7	5.9
D. barbeyi	flowers–full flower	0			3.4	0.1	3.5	7.6

[a] Stage of growth when larkspur was collected.
[b] Total consumption (g) by cattle or sheep during Latin square trials; air-dried weight for trials 2–4; fresh weight for trial 4.
[c] Probability of a greater F value for a feed (i.e., treatment) effect.
[d] No 3 × 3 trial was conducted.
[e] *D. barbeyi* was extracted with EtOH; the crude extract was added to alfalfa hay; the extracted larkspur residue was also tested; as one control, alfalfa hay had EtOH added, with EtOH allowed to evaporate; untreated alfalfa was also used.
[f] Composite larkspur treatments were made by collecting leaves or flowers from numerous plants in the same phenological stage. Plant was offered fresh and green daily to cattle.

TABLE 5. PEARSON CORRELATION COEFFICIENTS (r) AND PROBABILITIES ($P > |r|$) FOR RELATIONSHIPS AMONG CONCENTRATIONS (mg/g DRY WEIGHT) OF TOTAL ALKALOID, TOXIC ALKALOID, METHYLLYCACONITINE (MLA), AND 14-DEACETYLNUDICAULINE (14-DAN), AND AMOUNT OF LARKSPUR EATEN (g) IN TRIALS 2–4 BY CATTLE (N = 4, 7, and 4, RESPECTIVELY) USING VARIOUS LARKSPUR TREATMENTS[a]

	Total		Toxic		MLA		14-DAN	
	r	$P > r$	r	$P > r$	r	$P > r$	r	$P > r$
Trial 2								
Eaten (g)	0.87	0.12	−0.05	0.95	−0.04	0.96	−0.009	0.99
14-DAN	0.32	0.68	0.99	0.002	0.99	0.002		
MLA	0.31	0.69	0.99	0.0001				
Toxic	0.30	0.69						
Trial 3								
Eaten (g)	−0.18	0.69	−0.12	0.80	−0.12	0.80	−0.10	0.83
14-DAN	0.99	0.0001	0.99	0.0001	0.99	0.0001		
MLA	0.99	0.0001	0.99	0.0001				
Toxic	0.99	0.0001						
Trial 4								
Eaten (g)	−0.74	0.26	0.18	0.81	0.18	0.81	0.24	0.76
14-DAN	0.39	0.60	0.88	0.11	0.86	0.13		
MLA	0.53	0.47	0.99	0.0005				
Toxic	0.53	0.47						

[a]See Table 1.

Sheep Trials

Sheep displayed a distinct preference for the late-season plant material in all three tests. In the 4 × 4 test, they preferred the late-season *D. barbeyi* ($P < 0.01$) over the other three choices (Table 2). In the three-choice test, they also preferred the late season *D. occidentale* ($P = 0.05$) compared to *D. barbeyi* and *D. occidentale* collected in the vegetative stage (Table 2). When only two plant collections were compared in the 2 × 2 crossover tests, lambs showed no preference between *D. barbeyi* and *D. occidentale* in the vegetative stage. Thus, we accepted the hypothesis that total and/or toxic alkaloid concentration negatively influences sheep preference for tall larkspur. There was a strong tendency for sheep preferences to be negatively correlated with total and toxic alkaloid concentrations and to MLA concentration (Table 3). Both MLA and toxic alkaloid concentrations were related to each other and to total alkaloid concentration, but 14-DAN concentration was not related to the other alkaloid components (Table 3).

TABLE 6. CONSUMPTION OF INDIVIDUAL, FRESH *D. barbeyi* PLANTS DURING TRIALS 5–7 AND CONCENTRATION OF METHYLLYCACONITINE (MLA), 14-DEACETYLNUDICAULINE (14-DAN), TOXIC ALKALOID, AND TOTAL ALKALOIDS IN LEAVES AND FLOWERS FROM 2 REPRESENTATIVE STALKS FROM EACH PLANT

Trial/item	Plant phenology/characteristics	Consumption (bites)	$P > F^a$	Concentration (mg/g dry wt)			
				MLA	14-DAN	Toxic	Total
Trial 5: 4 × 4, cattle			0.60				
Plant 1	Full flower[b]/leaves	134		2.2	0.3	2.5	6.5
Plant 2	Early flower[c]/leaves	117		2.4	0.2	2.6	7.5
Plant 3	Full flower/leaves	251		4.4	0.2	4.6	6.2
Plant 4	Early flower/leaves	92		2.2	0.2	2.6	6.9
			—				
Plant 1	Full flower/flowers	0		2.5	0.2	2.7	5.8
Plant 2	Early flower/flowers	0		1.8	0.1	1.9	5.1
Plant 3	Full flower/flowers	0		2.0	0.1	2.1	6.0
Plant 4	Early flower/flowers	0		2.3	0.1	2.4	6.0
Trial 6: 4 × 4, cattle			0.88				
Plant 1	Full flower–shaded/leaves	182		2.7	0.2	2.9	3.9
Plant 2	Early flower–shaded/leaves	270		2.5	0.1	2.6	6.2
Plant 3	Full flower–no shade/leaves	245		2.7	0.1	2.8	3.6
Plant 4	Early flower–no shade/leaves	270		2.5	0.1	2.7	6.4

TABLE 6. Continued.

Trial/item	Plant phenology/characteristics	Consumption (bites)	$P > F^a$	Concentration (mg/g dry wt)			
				MLA	14-DAN	Toxic	Total
			—				
Plant 1	Full flower/flowers	0		4.7	0.3	5.0	7.9
Plant 2	Early flower/flowers	0		2.8	0.1	3.0	6.5
Plant 3	Full flower/flowers	0		2.4	0.1	2.6	7.5
Plant 4	Early flower/flowers	0		3.0	0.1	3.1	7.2
Trial 7: 4 × 4, cattle			0.54				
Plant 1	Full pod-no shade/leaves	114		2.1	0.04	2.2	3.1
Plant 2	Full pod-shaded/leaves	236		0.6	0.3	0.9	3.4
Plant 3	Early pod-shaded/leaves	140		1.3	0.1	1.4	4.1
Plant 4	Full pod-no shade/leaves	91		1.4	0.1	1.6	3.8
			0.26				
Plant 1	Full pod-no shade/pod	9		4.8	0.1	5.0	6.9
Plant 2	Full pod-shaded/pod	23		4.1	0.8	5.0	10.2
Plant 3	Early pod-shaded/pod	0		2.0	0.1	2.1	5.8
Plant 4	Full pod-no shade/pod	1		3.9	0.7	4.7	8.4

[a] Probability of a greater F value.
[b] Plants in full flower had fully elongated racemes with flowers fully opened.
[c] Plants in early flower had partially elongated racemes with flowers not fully opened.

TABLE 7. PEARSON CORRELATION COEFFICIENTS (r) AND PROBABILITIES ($P > |r|$) FOR RELATIONSHIPS AMONG CONCENTRATIONS (mg/g DRY WEIGHT) OF TOTAL ALKALOIDS, TOXIC ALKALOID, METHYLLYCACONITINE (MLA), AND 14-DEACETYLNUDICAULINE (14-DAN), AND TOTAL BITES BY CATTLE OF LEAVES AND REPRODUCTIVE PARTS DURING TRIALS 5–7 USING FRESH, CUT INDIVIDUAL LARKSPUR PLANTS (N = 24 FOR LEAVES AND REPRODUCTIVE PARTS)

	Total		Toxic		MLA		14-DAN	
	r	$P > r$	r	$P > r$	r	$P > r$	r	$P > r$
Bites	−0.32	0.13	−0.20	0.35	−0.17	0.42	−0.25	0.25
14-DAN	0.65	0.0006	0.53	0.008	0.38	0.06		
MLA	0.64	0.0007	0.98	0.0001				
Toxic	0.72	0.0001						

Cattle Grazing Trial

Cattle consumed 7 ± 2.5% of their overall diet as larkspur during the seven sampling dates. Larkspur consumption differed by dates, as cattle ate little larkspur during July, but increased larkspur consumption during mid- to late August (Figure 1). Most (>90%) of the larkspur consumed was leaves, with flowers and pods being selected only occasionally (data not shown). The concentration of toxic alkaloid in leaves declined slightly in late July and early August, but then increased slightly in mid- to late August (Figure 1). Total alkaloid concentration in leaves peaked in mid-July, decreased during July and much of August, but then increased in late August (Figure 1). On the three occasions (July 19, August 23 and 24) when cattle greatly increased leaf consumption during or just after storms, the toxic and total alkaloid concentration in leaves from preferred plants was 5.8 and 7.6, 1.8 and 5.4, and 2.3 and 6.0 mg/g, respectively, for the three dates. One of these samples contained a substantial amount (>5 mg/g) of toxic alkaloids, and two samples contained low to moderate (~2 mg/g) amounts of toxic alkaloids, and provided circumstantial evidence that toxic alkaloid concentration was not a major factor in selection of tall larkspur by grazing cattle. The leaf concentration of total alkaloids was in the range for most tall larkspur samples collected from this area and did not provide evidence for either avoidance or preference.

DISCUSSION

Our study indicates that concentrations of total or toxic alkaloid in tall larkspur generally have little or no influence on acceptability of tall larkspur to

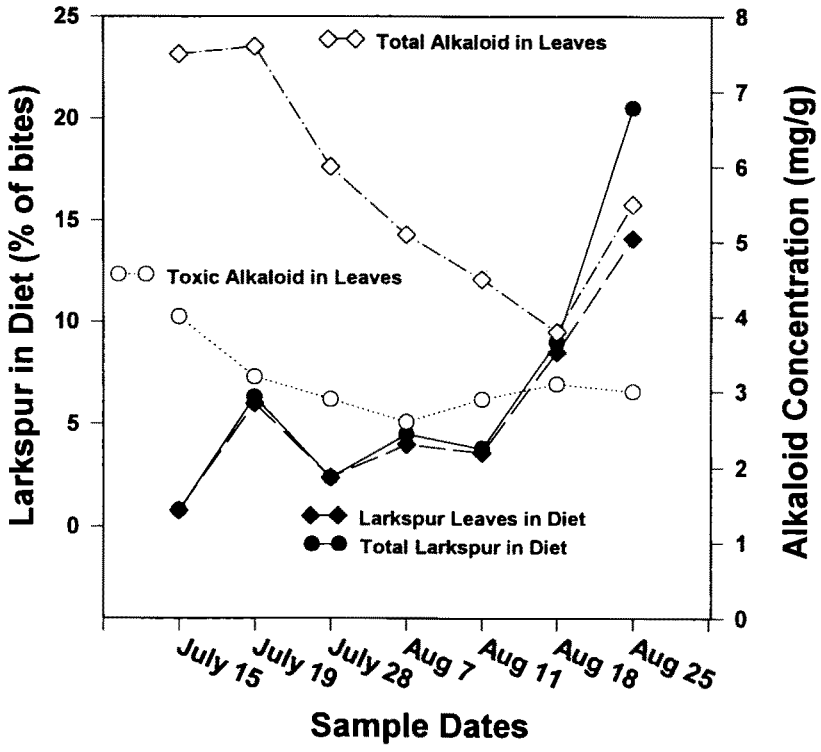

FIG. 1. Mean amounts of tall larkspur in diets of cattle (percentage of bites: leaves and total larkspur) and concentration (mg/g dry weight) of toxic alkaloids and total alkaloids in leaves sampled when cattle diets were determined during a grazing study near Yampa, Colorado, during summer, 1992.

cattle. The pen trials showed few or no relationships between alkaloid concentration (toxic or total) and amounts eaten by cattle, and the field grazing trial also indicated that toxic or total alkaloid concentrations in larkspur do not negatively influence consumption patterns. One collection of *D. barbeyi* from Utah contained no toxic alkaloids, yet had a total alkaloid concentration of nearly 39 mg/g. Cattle preferred this atypical plant collection during pen trials, thus providing strong circumstantial evidence that a high total alkaloid concentration is not deterrent to cattle. Conversely, this result indicates that very low toxic alkaloid concentrations may influence acceptability. Yet, we found no correlation in this or any other trial between toxic alkaloid concentration and amount eaten; further, other collections with lower toxic alkaloid concentrations (<2.0 mg/g) were not differentially accepted by cattle. We cannot explain the increase

in total alkaloids in leaves in late August during the grazing trial, but it seems doubtful that cattle increased larkspur consumption in response to this upswing in total alkaloid concentration. Our results are based primarily on correlation; hence, we can suggest causative factors but cannot be definitive about the interpretation. Plant factors other than alkaloids may have influenced our results (Reichardt et al., 1987).

There is disagreement on whether the taste of alkaloids affects palatability. Alkaloids apparently taste bitter to humans (DuPont et al., 1994) and livestock (Laycock, 1978), and bitterness may (Bate-Smith, 1972) or may not (Robinson, 1979; Glendinning, 1994) be universally repellent. Some classes of alkaloids apparently do not have a bitter taste (Molyneux and Ralphs, 1992). The alkaloid concentration of some toxic plants (e.g., *Phalaris* spp., *Lupinus* spp., *Senecio* spp.) is inversely related to palatability or may deter grazing (Ralphs and Olsen, 1987). Other alkaloid-containing toxic plants such as *Oxytropis* spp. and *Astragalus* spp., however, apparently do not show this relationship (Ralphs and Olsen, 1987).

Cattle did not eat enough larkspur in any trial for toxicosis to influence results. Nonetheless, aversive postingestive feedback may limit intake of specific foods (Provenza, 1995), and we have shown that larkspur ingestion by cattle above a toxic threshold is regulated by postingestive consequences (Pfister et al., 1990). Larkspur alkaloidal extracts can cause food aversions (Olsen and Ralphs, 1986), but cattle must consume about 9–11 mg of toxic alkaloids per kilogram of body weight for subclinical toxicosis to influence intake (Pfister et al., 1994b).

For reasons that are unclear, cattle in the pen and grazing trials near Yampa ate few or no flowers or pods. We have noted this same propensity in other grazing trials in this region during three other summers (Pfister, unpublished data). This is in contrast to cattle consumption patterns in Utah, where grazing trials have shown that cattle prefer larkspur pods from mature plants (Pfister et al., 1988a,b). We are presently unable to account for these differences in acceptability.

During trial 3 using alkaloid extract, cattle preferred the larkspur extract on alfalfa hay over the alkaloid-free residue, indicating that alkaloids did not deter consumption. Conversely, the extract-treated hay was less preferred than either untreated or EtOH-treated hay, suggesting a deleterious effect on acceptability. We recognize that extraction may remove many cellular constituents (e.g., some free sugars); however, 95% ethanol extraction only removes a small proportion of the major fructosans (Smith, 1969, 1973). It is possible that cattle selected the alkaloid-treated hay because of carbohydrates that were simultaneously extracted. Alternatively, it is possible that some very nonpolar constituents (e.g., terpenoids) were left in the residue, and acted as feeding deterrents to cattle.

In contrast to cattle, sheep consumption was negatively influenced by concentrations of total alkaloid and MLA in tall larkspur. Sheep apparently have a lower rejection threshold for bitter flavors than do cattle (Goatcher and Church, 1970), and this may account for their negative response to total alkaloid concentration in larkspur. Grazing sheep before cattle in tall larkspur-infested pastures has had limited success (Ralphs et al., 1991) because of the difficulty in getting sheep to eat larkspur before the plant is relatively mature. Our results suggest that higher concentrations of total alkaloid, or MLA, often found in immature larkspur, may be deterrent to sheep.

Arnold and Hill (1972) noted that the total alkaloid concentration of *Phalaris* spp. was more important to palatability than were individual alkaloids. Similarly, we found little indication that individual alkaloids influence cattle consumption; however, MLA concentration was negatively correlated with sheep consumption. Since MLA and total alkaloid concentration were also highly correlated, our sheep trials give no clear indication of the relative importance of total versus MLA alkaloid concentration.

At times there was not a strong correlation between concentrations of total and toxic alkaloids (or MLA concentration). Concentration of MLA was always related to toxic alkaloid concentration, since MLA comprises most of the toxic fraction, but MLA concentration was sometimes not related to amounts of 14-DAN. With plant material collected near Yampa, Colorado (trials 4–7), toxic alkaloid concentration was variable, whereas total alkaloid concentration was relatively low, with ratios of toxic to total alkaloid concentration varying from 0.3 to 0.75. Collections of tall larkspur from either Utah or Idaho (trials 1 and 2) had toxic/total ratios of about 0.2 or less. The extracted residue (trial 3) also did not have any toxic alkaloids, but had a small amount of total alkaloid left after extraction.

Even though we collected both shaded and unshaded larkspur plant for comparisons in trials 6 and 7, no distinct trend emerged for alkaloid concentration as leaves from both shaded and unshaded plants had total alkaloid concentrations averaging about 4 mg/g and toxic alkaloid concentrations averaging about 2 mg/g. The alkaloid concentrations in flowers also did not show clear differences due to shading. We have previously found that grazing cattle prefer to graze shaded larkspur plants over plants growing in sun (Pfister et al., 1988a), but that trend was not evident in this study.

Overall, this study indicates that knowledge of the concentration of total or toxic alkaloid in tall larkspur will not give any clear indication of whether cattle are likely to ingest the plant or not. We can make accurate predictions about the toxicity of tall larkspur based on the concentration of toxic alkaloids, but this information will only provide the livestock producer with the risk of toxicity assuming some level of consumption. Predicted consumption by cattle must necessarily be based on an understanding of other factors, such as plant

phenology (Pfister et al., 1988a,b), or weather patterns (Ralphs et al., 1994), but not plant toxicity.

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SEX PHEROMONE OF APHID PARASITOID *Praon volucre* (HYMENOPTERA, BRACONIDAE)

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Abstract—Mating in the aphid parasitoid *Praon volucre* is mediated by a female-produced sex pheromone, which laboratory behavioral studies suggest is released from the abdomen. Gas chromatography coupled with electrophysiological and behavioral assays on males showed one area of activity in an air entrainment sample of virgin females. However, levels of pheromone production are very low and this has so far precluded its identification.

Key Words—Hymenoptera, aphid, parasitoid, sex, pheromone, GC, EAG, behavior.

INTRODUCTION

Praon volucre Haliday (Hymenoptera, Braconidae) is a generalist parasitoid attacking several species of aphid (Homoptera, Aphididae) and has been used in biological control programs since the late 1950s (Stary, 1970). More recently, it has been shown that *P. volucre* females are attracted by aphid sex pheromones, and this innate response may be utilized to manipulate *Praon* populations in the field with a view to improving aphid control (Powell et al., 1993; Lilley et al., 1994). However, to develop an integrated strategy for aphid management, there is a need for accurate monitoring systems for both aphids and their natural enemies. Parasitoid sex pheromones can provide a method of assessing natural

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or released populations of these beneficial insects (Brodeur and McNeil, 1994). Laboratory and field studies have supplied evidence for sex pheromone production in virgin female *P. volucre* (Decker et al., 1993) and preliminary studies on chemical identification are reported here.

METHODS AND MATERIALS

Insects. P. volucre were maintained on a clone of the English grain aphid, *Sitobion avenae* (Fab.), reared on oats, *Avena sativa* L. (Poaceae), at 18°C with a 16L : 8D hr photoperiod. Parasitoid mummies (pupal stage) were collected from culture cages and isolated in corked glass tubes. After emergence, mummies were removed from the tubes and the parasitoids sexed and provided with an aqueous solution of honey.

Pheromone Collection—Crude Extracts. Males and virgin females (1–2 days old) were anaesthetized by chilling and then extracted in diethyl ether for 1 hr. Preliminary experiments indicated that the age of the females (1, 2, or 3 days old) did not affect the activity of the extracts. To determine the location of pheromone production, 1-day-old virgin females were dissected, and the head, thorax (including the wings and legs) and abdomen were extracted separately with ether.

Pheromone Collection—Air Entrainment. Twenty virgin females (1–3 days old) were placed in a glass culture vessel (Quickfit FV range, 1 liter) and air, dried and purified by passage through activated 5 Å molecular sieve and charcoal, was drawn at 1 liter/min through the vessel. Volatiles were entrained over a 24-hr period onto Porapak Q that had been purified by washing with diethyl ether (5 ml) and heating for 12 hr at 150°C under a stream of nitrogen. Collected volatiles were desorbed from the Porapak by elution with freshly distilled diethyl ether. The resulting extract was concentrated under a stream of nitrogen and stored in sealed glass ampoules at −20°C.

Electrophysiology. Electroantennogram (EAG) recordings from 1-day-old male parasitoids were made using Ag–AgCl glass electrodes filled with saline solution [composition as in Maddrell (1969) but without the glucose]. The insect was anesthetized by chilling, and the head was excised and mounted on the indifferent electrode. The tip of the recording electrode was removed so that its inside diameter was just wide enough to accept the uncut terminal process of the antenna. The signals were passed through a high impedance amplifier (UN-03b, Syntech) and displayed on an oscilloscope.

Coupled Gas Chromatography (GC)–Electrophysiology. The coupled GC-electrophysiology system, in which the antennal preparation is directly coupled to the capillary column GC, has been described previously (Wadhams, 1990). Separation of the air entrainment sample was achieved on an AI 93 GC equipped

with a cold on-column injector and a flame ionization detector (FID). The column (30 m × 0.53 mm ID, HP-1) was maintained at 40°C for 2 min and then programmed at 10°/min to 250°C. The carrier gas was hydrogen. The outputs from the EAG amplifier and the FID were monitored simultaneously on a chart recorder.

Coupled GC-Behavioral Assay. The effluent from the GC column was split equally between the FID and a transfer line, the end of which was inserted through a hole into a tube connected to a glass chamber containing a single 1-day-old *P. volucre* male. An airstream (600 ml/min) carried the effluent to the insect. The insect and the chart recorder monitoring the signal from the FID of the GC were simultaneously video recorded. Wing-fanning responses were converted into bins that were cumulated over 15 sec and marked onto the GC trace to provide response profiles. Individual records were time-synchronized on the injection time. Elution times of the compounds in the five analyses were reproducible, so that all records could be cumulated without further processing. The test sample and GC conditions were the same as for the coupled GC-EAG.

Olfactometry. The behavioral responses of males were observed in a Pettersson olfactometer (diameter 10 cm) having a weak airstream (600 ml/min) directed towards the center from each of four side arms (Pettersson, 1970). The test stimulus was placed at the end of one of the arms. Treatments were: (1) 1-day-old virgin females, (2) 1-day-old males, (3) the crude extract of virgin females (0.1 female equivalents) and (4) the air entrainment sample of virgin females (5 female-hour equivalents). The olfactometer was placed in a darkened room and illuminated from the top. A 1-day-old naive male *P. volucre* was allowed to enter the chamber through a hole (4 mm) in the center of the olfactometer floor. The time spent in each olfactometer arm and the first choice made by the insect were recorded by means of the computer program OLFA 1.0 (Nazzi, 1995). The experiments lasted 15 min, and the olfactometer was rotated through 90° every minute. After each experiment, the olfactometer was thoroughly washed in 1% aqueous anionic detergent (Teepol) and rinsed with water. The experiments were carried out between 11:00 and 14:00 hr and replicated a minimum of seven times.

Wing-Fanning Bioassay. In previous studies, male wing fanning was a key behavioral component of the mating sequence in aphid parasitoids (Stary, 1970) and its occurrence provides strong evidence for the presence of a sex pheromone (Askari and Alishah, 1979; Decker et al., 1993; McNeil and Brodeur, 1995). Consequently, the wing-fanning bioassay was used to confirm the presence of a sex pheromone and to determine the source of pheromone production. The test sample (5 μl) was applied to a strip of filter paper (Whatman No. 1; ca. 0.5 × 2.0 cm), which was inserted, after evaporation of the solvent, into a glass tube (0.8 × 3.0 cm) containing a 1-day-old naive male parasitoid. The wing fannings that occurred immediately after introduction of the stimulus and

over the next 60 sec were recorded. Treatments were: (1) the crude extracts of males and virgin females (0.1 insect equivalents), (2) extracts of female head, thorax, and abdomen, and (3) the air entrainment sample of virgin females (5 female-hour equivalents).

Statistical Analysis of Data. The times spent in the treated and control arms of the olfactometer were analyzed using a paired t test. The number of insects that chose the treated arm first, out of the total number of males tested, was compared with the figure expected in the case of a binomial distribution. The results of the wing-fanning bioassay were analyzed, when necessary, with the Fisher exact solution test.

RESULTS AND DISCUSSION

In the olfactometer, males showed significant responses to live virgin females in terms of first choice (Table 1) and total time spent in the treatment arm (Table 2). Crude whole body extracts of virgin females (0.1 insect equivalents) and the air entrainment sample from virgin females (5 female-hour equivalents) were also attractive to males (Tables 1 and 2). No response was observed to live males.

In the wing-fanning bioassay, the whole-body extracts of virgin females and the air entrainment sample elicited responses from over 80% of the males tested, but no response was obtained to the male extract (Table 3). The wing-fanning bioassay was also used to determine the site of pheromone production. Of the 15 males tested, 13 responded strongly to the extract of female abdomens, but the head extract showed no activity. The thorax extract was weakly active, with three of 15 males responding; this may be due to contamination of the wings, which are normally folded above the abdomen. In addition, the marked

TABLE 1. RESPONSE OF *Praon volucre* NAIVE MALES TO DIFFERENT STIMULI IN OLFACTOMETER: TREATED ARM CHOSEN FIRST

Stimulus	Males tested (N)	Males choosing treated arm (N)	Probability
Control (ether)	7	3	NS
Live male	8	3	NS
Live virgin female	9	5	<0.05
Crude extract (0.1 female equiv)	11	6	<0.05
Air entrainment (5 female hr equiv)	9	6	<0.01

NS = not significantly different from control at $P = 0.05$.

TABLE 2. RESPONSE OF *Praon volucre* NAIVE MALES TO DIFFERENT STIMULI IN OLFACTOMETER: MEAN TIME SPENT IN TREATED OR CONTROL ARMS

Stimulus	Males tested (*N*)	Mean time (min ± SE) spent in		Probability
		treated	control	
Control (ether)	7	2.3 ± 1.7	1.4 ± 0.6	NS
Live male	8	3.7 ± 1.7	2.6 ± 0.7	NS
Live virgin female	9	6.7 ± 1.1	1.1 ± 0.5	<0.01
Crude extract (0.1 female equiv)	11	6.0 ± 1.9	1.2 ± 0.5	<0.05
Air entrainment (5 female hr equiv)	9	6.8 ± 1.8	1.2 ± 0.4	<0.05

NS = not significantly different from control at $P = 0.05$.

grooming behavior of parasitoids could play a role in spreading pheromone over other parts of the body. Thus, pheromone production, as with *Diaeretiella rapae* (Askari and Alishah, 1979) and *Aphidius nigripes* (McNeil and Brodeur, 1995), appears to be associated with the female abdomen.

EAG responses from male antennae were recorded to groups of 10 live virgin females (0.26 ± 0.02 mV; $N = 5$) and to 50 female-hour equivalents of the air entrainment sample (0.5 mV), suggesting that this sample is a realistic reflection of the level of volatiles produced by the females. Coupled GC-EAG showed only one major area of activity, corresponding to a minor component in the sample (Figure 1). The activity of this component was confirmed using the coupled GC-behavioral assay: three of the five males tested showed wing-fanning responses in the area associated with the electrophysiologically active peak (Figure 1). One of the three and one other male showed wing-fanning behavior in two areas at shorter GC retention times, but these were not associated with electrophysiological activity.

TABLE 3. RESPONSE OF *Praon volucre* NAIVE MALES TO DIFFERENT STIMULI IN WING-FANNING BIOASSAY

Stimulus	Males tested (*N*)	Males responding (*N*)
Control (ether)	48	0
Crude extract (0.1 male equiv)	30	0
Crude extract (0.1 female equiv)	48	40
Air entrainment (5 female hr equiv)	48	42

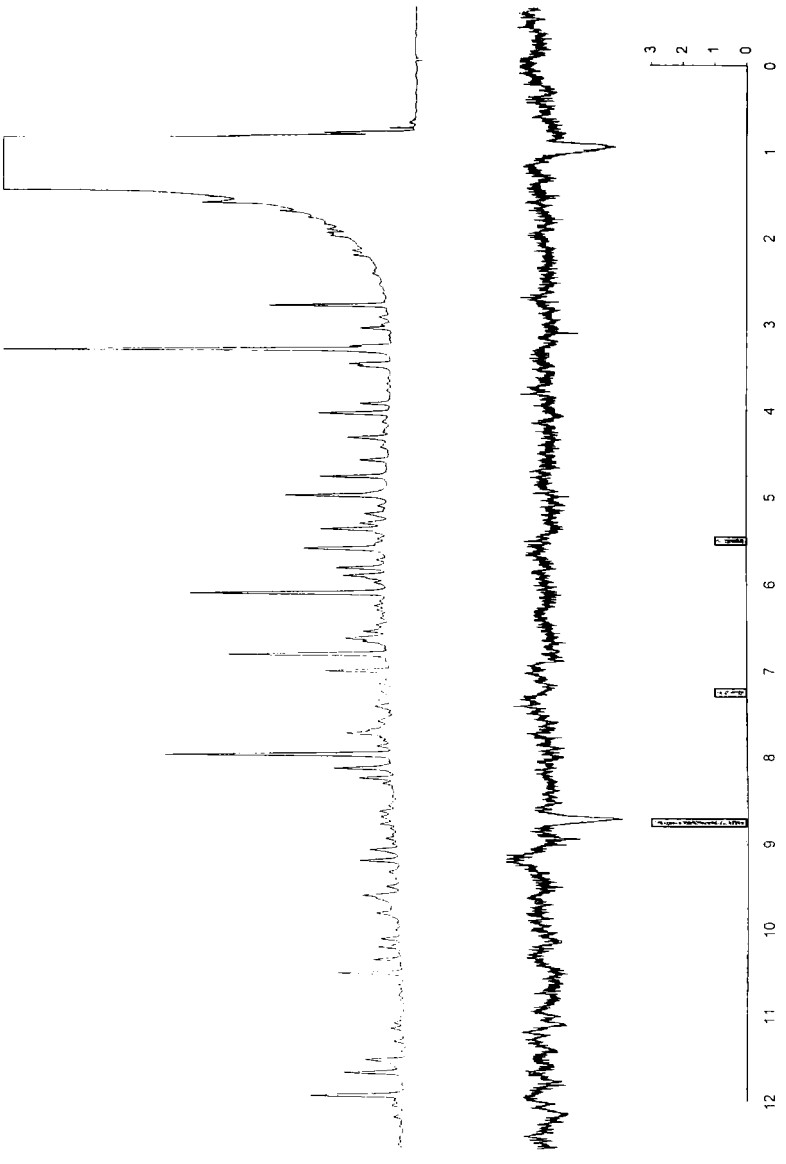

FIG. 1. *Praon volucre* males: coupled GC-EAG and GC-behavioral assay with the air entrainment sample of virgin females (50 female-hour equivalents). Upper trace: GC of volatiles from virgin females. Middle trace: EAG response of one male. Lower trace: behavioral responses; vertical axis shows number of males, out of five tested, exhibiting wing-fanning activity

This study has confirmed a female sex pheromone for *P. volucre*, although the amount of pheromone produced is very small. Approximate quantifications from the GC peak area of the air entrainment sample suggest that pheromone production is of the order of only picograms per hour. The similarity between the EAG responses of males to live virgin females and to the entrainment sample suggests that this is not an experimental artifact. Although males are shown to be highly sensitive to the pheromone, with as little as 5 female-hour equivalents eliciting a response in the olfactometer and 10 virgin females per trap attracting males in the field (Decker et al., 1993), the paucity of pheromone has so far precluded its identification.

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SENSITIVITY OF *Lemna* BIOASSAY INTERACTS WITH STOCK-CULTURE PERIOD

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Abstract—The effect of stock-culture period on the sensitivity of the *Lemna* bioassay to four different phenolic substances was evaluated in this study. The sensitivity of the bioassay interacted with the stock-culture period of either 11, 14, or 18 days. After a seven-day test *p*-hydroxybenzoic acid (HBA), vanillic acid (VA), *trans*-cinnamic acid (CA), and ferulic acid (FA) with a concentration of 0.05 mM significantly reduced the number of fronds and the dry weight compared with the untreated control when the stock-culture period had been exactly 14 days. The sensitivity after the shorter (11 days) or longer (18 days) stock-culture period was reduced, and the differences in the dry weight to the untreated control were not significant after a stock culture period of 18 days. The two higher concentrations (0.1 and 0.25 mM) showed stronger inhibition. A comparison of the inhibition at 0.05 mM revealed that the stock-culture period affected the relative toxicity of the four phenolic substances. Since the pH increased in the stock-culture flasks during the 18-day period from 6.25 to 7.9, we hypothesize that differences in the *Lemna* assay can be at least partly attributed to a pH effect, possibly in combination with a relative nutrient deficiency. We recommend a standardization of the stock-culture period to 14 days to improve the sensitivity of the *Lemna* bioassay.

Key Words—*Lemna minor*, duckweed, bioassay, sensitivity, phenols, stock-culture period, pH.

INTRODUCTION

Duckweeds (*Lemna* spp.) are small floating angiosperms common to a wide range of rivers and lakes all over the world (Hillman, 1961). Beside its use in

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the field of water purification, various *Lemna* species have gained broad acceptance for bioassays in ecological and allelopathy research due to their easy handling as well as their sensitivity to a wide range of toxic substances. *Lemna* species such as *L. minor*, *L. obscura*, and *L. gibba* are extremely sensitive to various phenolic substances (Einhellig et al., 1985; Ramirez Toro et al., 1988), different pesticides (Lockhart et al., 1983, 1989; Cowgill et al., 1989; Cooley and Foy, 1992; Grossmann et al., 1992), alkaloids (Wink and Twardowski, 1992), quinones (Einhellig and Souza, 1992; Hejl et al., 1993), heavy metals (Jenner and Janssen-Mommen, 1993), mycotoxins (Vesonder et al., 1992), as well as mixtures with unknown chemicals from soil extracts, plants, or leachates from polluted sediments, etc. (e.g., Jenner and Janssen-Mommen, 1989; May and Ash, 1990; Elakovich and Wooten, 1991; Clement and Bouvet, 1993).

The most common measurement of such phytotoxic activity has been the number of *Lemna* fronds after a period of growth, but individual dry weight, growth rate, or changes in the chlorophyll content have also been determined regularly (Einhellig et al., 1985, Wink and Twardowski, 1992). Chen and Leather (1990) quantified effects on chlorophyll content per frond in *L. minor* and the anthocyanin content in *L. obscura* as part of their investigation of the phytotoxicity of the sesquiterpene lactone artemisinin. In order to gain insight into the physiological action of suspected phytotoxic substances, variables such as photosynthesis and respiration (Stiles et al., 1994) or protein synthesis (Cooley and Foy, 1992) have been monitored.

Despite proposals of Leather and Einhellig (1988), the protocols in experiments using the *Lemna* bioassay often vary considerably. This makes comparisons of results extremely difficult with respect to the toxicity of various compounds. In most protocols, the particular *Lemna* plant is chosen based on the developmental stage, i.e., three or four frond colonies. The preculture period has been documented only in a few cases where *Lemna* species were the bioassay. Clement and Bouvet (1993) mention that in their experiments the preculture period lasted exactly 14 days; however, no details were given as to why this period was originally chosen. In preliminary experiments with the *Lemna* bioassay following Einhellig et al. (1985), we experienced unexpected results. Since no known cause could explain them and most papers do not provide information about the preculture period, we investigated the interaction between the preculture period of *Lemna minor* and its sensitivity in a bioassay.

We present results of experiments evaluating the interaction between phytotoxic effects of various phenolic substances and the preculture period of *L. minor*. We used phenolic substances in this experiment, since extensive research has identified the chemical nature of allelochemicals associated with plant-soil-microbial systems as being mainly phenolic substances (Siqueira et al., 1991).

METHODS AND MATERIALS

Culture Conditions. Axenic cultures of duckweed, *Lemna minor* strain 5, were grown in autoclaved, cotton-closed 100-ml Erlenmeyer flasks with 50 ml of E medium (Cleland, 1979). The stock cultures were maintained for either 11, 14, or 18 days in an environmental growth chamber at 28°C under constant cool white light (170 $\mu E/m^2/sec$).

Bioassay. The bioassay was performed according to the methods described previously by Einhellig et al. (1985). All procedures were performed on a clean bench with aseptic techniques. Fresh E medium was prepared for each bioassay. E medium (1.5 ml) was dispensed into each well of a sterile 24-well tissue culture plate with a sterile pipet. Phenolic substances dissolved in 99% methanol were applied by dispersing 5 μl with a Eppendorf Varipette 4710 into each well with two complete tissue culture plates for each phenolic compound, i.e., 12 replicates in different wells, which were arranged randomly on the plates.

Concentrations of 0.05 mM, 0.1, and 0.25 mM of *p*-hydroxybenzoic acid (HBA), vanillic acid (VA), *trans*-cinnamic acid (CA), or ferulic acid (FA) were tested. The control received also 5 μl of methanol, giving a total of 24 tissue culture plates (576 separate wells) for the entire experiment. *Lemna* colonies at the three-frond developmental stage were transferred by a flamed culture loop into each well. The culture plates were covered and placed in an environmental growth chamber with conditions given above. The plates were arranged randomly, and their position in the growth chamber was changed twice every day to minimize effects of light, temperature, and/or relative humidity differences. In order to allow for better gas exchange and reduce evaporation of culture medium, the lids of the culture plates were lifted every day for 5 min on the clean bench.

Harvest. After exactly seven days, *L. minor* was carefully removed from each well of the culture plate and placed into water for a frond count. The fronds were then air dried for two days, and the dry weight was determined on a balance accurate to 0.01 mg.

Data Analysis. The comparisons between treatments were analyzed using a general linear model (GLM).

RESULTS

L. minor was more sensitive to the two hydroxybenzoic acids *p*-hydroxybenzoic acid (HBA) and vanillic acid (VA), than to the cinnamic acids *trans*-cinnamic acid (CA) and ferulic acid (FA) (Figure 1). The application of *trans*-cinnamic acid was most inhibitory and caused bleaching at 0.1 mM and death

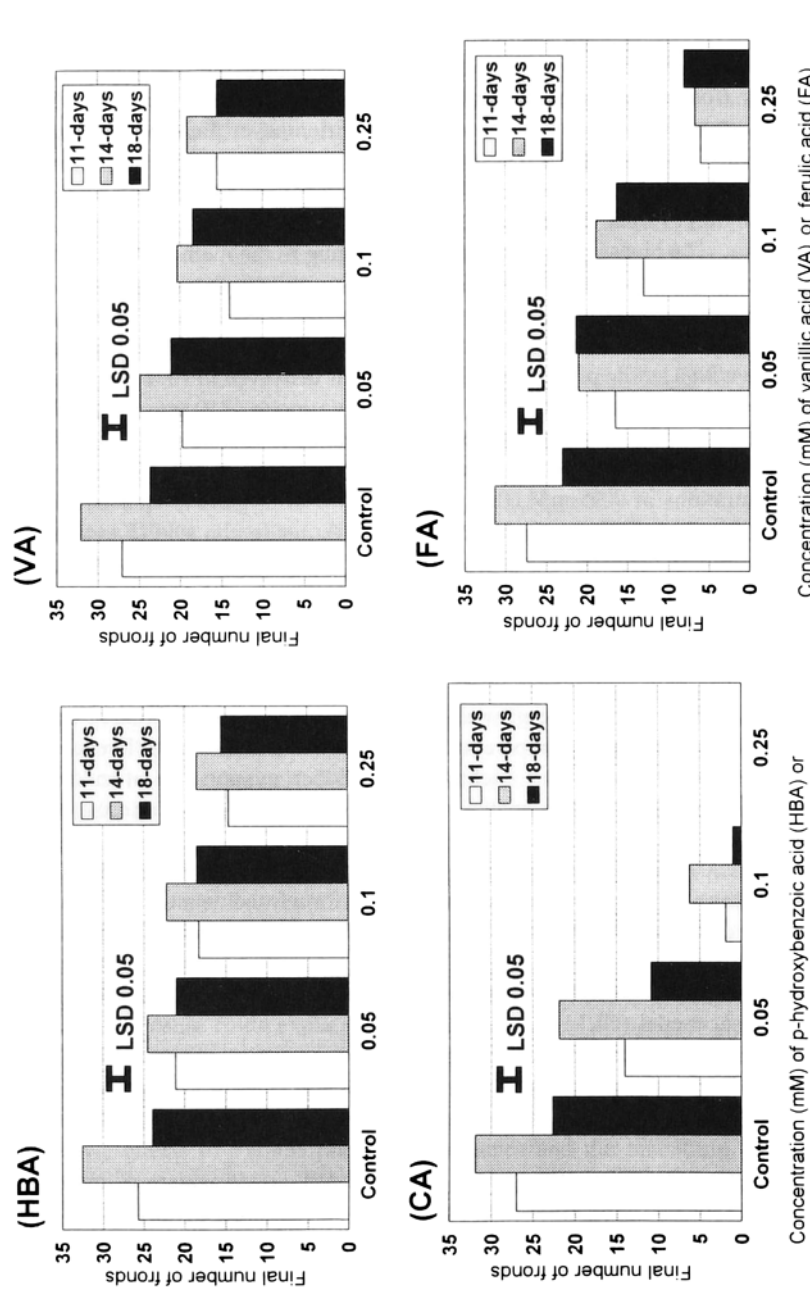

FIG. 1. Effect of stock-culture period of 11, 14, or 18 days and *p*-hydroxybenzoic acid (HBA), vanillic acid (VA), *trans*-cinnamic acid (CA), or ferulic acid (FA) on the final frond number of *L. minor* grown for seven days in tissue culture wells.

of *L. minor* at 0.25 mM. Regardless of the concentration of the test solution, in all but two cases *L. minor* produced the largest number of fronds after a stock-culture period of 14 days (Figure 1). Differences in results among the four phenolic compounds tested were inconsistent in relation to stock-culture periods of 11 or 18 days.

After a seven-day exposure period, the number of fronds from *L. minor* was significantly reduced by all four phenolic substances at 0.05 mM when the stock-culture period was 14 days.

The dry weight of *L. minor* as influenced by the phenolic substances was similar (Figure 2). Each phenolic compound caused a significant reduction in dry weight at a concentration of 0.05 mM after a 14-day stock-culture period. However, the differences were smaller between the response at 0.05 mM compared with the higher concentrations of 0.1 or 0.25 mM in reduction in the number of *L. minor* fronds, suggesting that the latter is more suitable for such comparisons. As noted for the number of fronds, the dry weight of *L. minor* interacted with the stock-culture period. However, in contrast to the results shown for the frond numbers, the plant weight was in most cases larger after a stock-culture period of 11 days. The only exceptions were the control treatments. Significant differences between the control treatments and the 0.05 mM concentration of the different phenols occurred only after a stock-culture period of 11 or 14 days.

A comparison of the mean squares and the F values of the analysis of variance for the number of fronds and the dry weight of *L. minor* reveals the significant effect of the stock-culture period as well as the two- and three-way interactions of the stock-culture period with the other factors tested in this experiment (Table 1).

Although the differences seemed to be small, a direct comparison of the number of fronds and the dry weight responses of *L. minor* exposed to the four phenolic substances at the lowest concentration of 0.05 mM showed an interaction with the stock-culture period. These results demonstrate the importance of interactions with the stock-culture period at low concentrations of a phytotoxic substance (Tables 2 and 3).

L. minor produced nearly the same numbers of fronds per tissue culture well at a concentration of 0.05 mM *p*-hydroxybenzoic acid (HBA) regardless of whether the stock-culture period lasted 11 or 18 days. The response towards ferulic acid (FA) was clearly modified, however, and the number of fronds was 23% lower after a stock-culture period of only 11 days compared with 18 days. The largest relative differences in sensitivity at 0.05 mM in relation to the stock-culture period occurred in the case of *trans*-cinnamic acid (CA), for dry weight and number of fronds at all three stock-culture periods (Tables 2 and 3). Different rankings of the toxicity of the four phenolic substances occurred at the

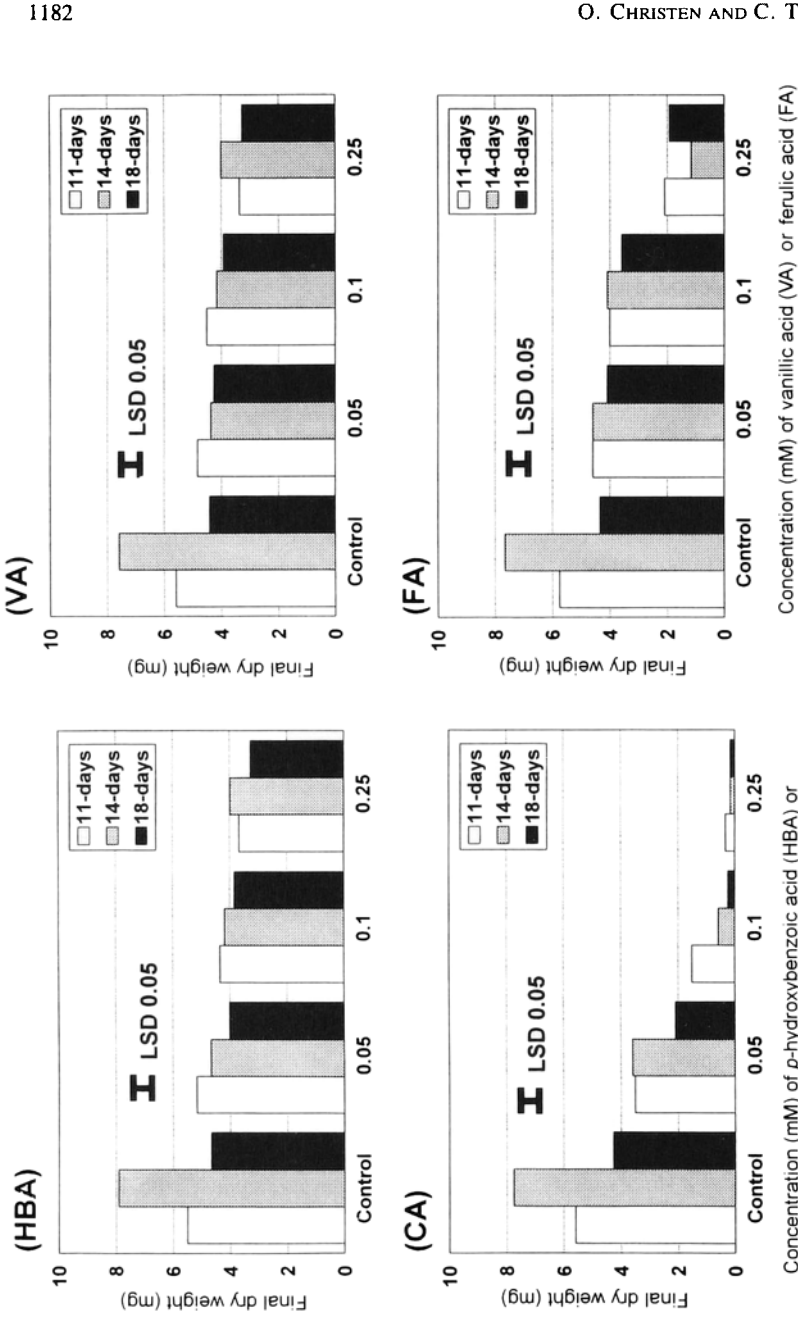

FIG. 2. Effect of stock-culture period of 11, 14, or 18 days and *p*-hydroxybenzoic acid (HBA), vanillic acid (VA), *trans*-cinnamic acid (CA), or ferulic acid (FA) on the final dry weight (mg) of *L. minor* grown for seven days in tissue culture wells.

TABLE 1. ANALYSIS OF VARIANCE FOR NUMBER OF FRONDS AND FINAL DRY WEIGHT OF *L. minor* IN TISSUE CULTURE WELLS

Source	*df*	No. of Fronds		Final dry weight (mg)	
		Mean square	*F* value	Mean square	*F* value
Phenol (P)	3	3042.7	460.5[a]	140.1	5053.1
Concentration (C)	3	8163.5	1235.5	342.0	12329.7
P × C	9	549.8	83.2	25.0	903.3
Stock-culture period (S)	2	1223.5	185.1	64.4	2322.0
P × S	6	48.5	7.3	0.6	23.4
C × S	6	142.5	21.5	29.6	1067.9
P × S × C	18	26.6	4.0	1.0	37.9
Error	575	6		0.02	

[a] All main factors are assumed to be fixed. They and the interactions are significantly different at $P < 0.001$.

concentration of 0.05 mM. Such a modification of the inhibition of the growth or development of *L. minor* by the stock-culture period occurred at all tested concentrations. The relative differences, however, were smaller and, therefore, in the cases of 0.1 or 0.25 mM did not cause a variation in the ranking of the four substances according to their toxicity.

TABLE 2. EFFECT OF STOCK-CULTURE PERIOD AND FOUR DIFFERENT PHENOLICS ON FINAL FROND NUMBER OF *L. minor* GROWN FOR SEVEN DAYS IN TISSUE CULTURE WELLS[a]

Stock-culture period (days)	Phenolic (0.05 mM)[b]			
	HBA	VA	CA	FA
11	21.6	19.8	14.0	16.5
	(1)	(2)	(4)	(3)
14	24.5	24.9	21.8	21.0
	(2)	(1)	(3)	(4)
18	21.0	21.1	10.8	21.3
	(3)	(2)	(4)	(1)

[a] Ratings according to the frond number are given in parentheses. $LSD_{0.05} = 2.05$.
[b] Abbreviations: *p*-hydroxybenzoic acid, HBA; vanillic acid, VA; *trans*-cinnamic acid, CA; and ferulic acid, FA.

TABLE 3. EFFECT OF STOCK-CULTURE PERIOD AND FOUR DIFFERENT PHENOLICS AT FINAL DRY WEIGHT (mg) OF *L. minor* GROWN FOR SEVEN DAYS IN TISSUE CULTURE WELLS[a]

Stock-culture period (days)	Phenolic (0.05 mM)[b]			
	HBA	VA	CA	FA
11	5.16	4.83	3.50	4.66
	(1)	(2)	(4)	(3)
14	4.66	4.37	3.58	4.58
	(1)	(3)	(4)	(2)
18	4.00	4.25	2.08	4.08
	(3)	(2)	(4)	(2)

[a] Ratings according to the dry weights are given in parentheses. $LSD_{0.05} = 0.42$.
[b] Abbreviations as in Table 2.

DISCUSSION

The *Lemna* bioassay has proved to be a powerful tool to monitor the biological activity of a large range of potentially phytotoxic substances. Following the protocol given by Einhellig et al. (1985), it is possible to evaluate and compare the toxicity of extremely small samples of test solution.

All four phenolics used in our experiment could be detected at a concentration as low as 0.05 mM. In contrast, Einhellig et al. (1985) reported a threshold of 0.25 mM for ferulic acid in *L. minor*, and Ramirez Toro et al. (1988), using *L. gibba*, again established a threshold for ferulic acid of 0.75 mM. The results of our experiment demonstrate that the reason for such a difference in the sensitivity of the *Lemna* bioassay might be differences in the stock-culture period.

The reason for such a strong negative effect of the long stock-culture period of 18 days on the sensitivity of the bioassay might be the increase in the pH of the stock solution over time. We have monitored the pH in the different treatments and measured pH at 6.25 after 11 days, 6.89 after 14 days, and 7.90 after 18 days. Einhellig et al. (1985) report no growth reduction when the pH of the medium was in the range between 4.0 and 6.0. Therefore an effect of pH 7.90 of the stock-culture solution on the sensitivity of the *Lemna* bioassay seems possible, even when the pH in the solution for the bioassay period was adjusted to 4.6. A second factor that we did not control in our experiment might have been a relative nutrient deficiency in the stock-culture flask after the long stock-culture period of 18 days. This hypothesis concurs with results from Cowgill et al. (1989), who compared the effect of a herbicide on *L. minor* for either seven or 14 days. They report less sensitivity for the shorter bioassay

period and attribute this to nutrient deficiencies in the longer experiment. Such a prolonged effect of various stock-culture periods with different pH and/or nutrient status might be explained by possible changes in the hormonal balance of the *Lemna* plants. As Thorsteinsson and Eliasson (1990) demonstrated in experiments with *L. gibba*, the reason might be changes in the cytokinin levels, which respond slowly to an improved nutritional situation. However, the reason for the described effects remains open to discussion until more detailed physiological studies have been conducted.

An explanation for the reduced sensitivity of the *Lemna* bioassay after an 11-day stock-culture period is less obvious, since differences of the pH in the stock-culture solution and/or nutrient deficiencies are not the explanation. We argue that after an 11-day stock-culture period, *Lemna* plants are less sensitive because they have not reached the developmental stage to separate so easily in different new rosettes. This hypothesis is confirmed by the observation that the average dry weight per frond was 0.26 mg for *Lemna* plants after an 11-days stock-culture period, whereas it was only 0.18 or 0.19 mg after 14 or 18 days, respectively. Whether this explanation is sufficient requires further developmental studies.

CONCLUSION

Our results demonstrate that a standardization to 14 days for the stock-culture period before using the *Lemna* bioassay greatly enhances its sensitivity and makes it possible to detect even smaller concentrations of phytotoxic substances. Additionally, in order to ensure comparability of results of the *Lemna* bioassay among different experiments, a strict standardization of the stock-culture period and/or monitoring of the pH in the stock-culture solution is desirable. We recommend a 14-day stock-culture period. This would be a further step to improve the *Lemna* bioassay for the field of allelopathic research and make it possible to employ it for the detection of potential phytotoxic substances at extremely small concentrations.

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CHEMOECOLOGICAL ROLE OF MAMMALIAN URINE IN HOST LOCATION BY TSETSE, *Glossina* spp. (DIPTERA: GLOSSINIDAE)

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Abstract—Trap catch size was used to investigate whether *Glossina pallidipes* and *G. longipennis* could distinguish between the urine of the African buffalo, cattle, and waterbuck at Nguruman in southwest Kenya. NG2G traps baited with aged urine of these bovids caught significantly more of each tsetse than did the controls. The mean catch of either tsetse species in traps baited with aged urine of buffalo and cattle (tsetse hosts) and waterbuck (a nonhost) did not differ significantly. Aged urine from both tsetse hosts (buffalo and cattle) and the nonhost (waterbuck) was found to contain 4-cresol and 3-*n*-propylphenol in about the same ratio. However, the aged urine from other tsetse hosts (bushpig and warthog) lacked 3-*n*-propylphenol. Cattle urine had to be aged outside the soil to produce statistically significant increases in the trap catch of *G. pallidipes*. Furthermore, patches of soil on which fresh urine of cattle was deposited, and in which it was aged, failed to effect a significant increase in the trap catch of either *G. austeni*, *G. brevipalpis*, or *G. pallidipes* as Gazi, southeast Kenya. The likelihood of tsetse either differentiating its hosts from nonhosts or locating favored hosts by the urine scent appears remote under natural conditions. It is more likely that the chemical signals critical for host location by the tsetse emanate from skin glands rather than volatilize from the urine of mammals.

Key Words—Tsetse, *Glossina*, host location, mammalian urine, phenols, attraction, nonhosts.

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INTRODUCTION

Ecosystems in which tsetse flies thrive necessarily also support various species of mammals, including hosts and nonhosts of tsetse. Every two to five days of their adult lives, tsetse must locate hosts from which to suck blood (Jackson, 1954; Glasgow, 1963; Randolph and Rogers, 1978; Madubunyi, 1989) or else incur reproductive loss through aborted pregnancies and protracted interlarval periods (Saunders, 1960, 1972) and/or perish from starvation. In nature, female tsetse are not always able to produce enough offspring to ensure survival of the species (Jordan, 1986). Precisely timed and adequately sized blood intake by the female during each 10-day pregnancy cycle is imperative for the production of each viable offspring (Mellanby, 1937) and indispensable for maintaining net population growth of tsetse. Timely location of mammalian hosts at the crucial moments of the trophic cycle is thus fundamental to the survival of tsetse species.

Most mammals maintain their respective identities, social structures, reproductive behavior, community cohesion, etc., through visual, auditory, tactile, and olfactory communication systems. Olfactory communication encompasses generation of chemical signals by a presumptive sender and their transmission (generally as airborne volatiles) to a presumptive receiver, who responds appropriately to them (Eisenberg and Kleiman, 1972). These chemical signals are by-products of metabolism, found in the excretory products (e.g., urine, feces, sweat and breath), or glandular secretions and exudations of mammals.

The ability to intercept the chemical signals with which the hosts exchange vital intraspecific information would confer major selective advantage to a genus of obligate blood-sucking arthropods such as *Glossina* Weidemann. By suggesting that *G. pallidipes* Austen uses its olfactory sense to locate hosts, Fuller and Mossop (1929) implied that tsetse has such ability. Lloyd (1935) further confirmed that *G. swynnertoni* Austen could perceive airborne volatiles from Zebu cattle (*Bos* spp.) that were not within sight and at a distance of 23 m. Bax (1937) later reported that the scent of ox manure was unattractive to *G. swynnertoni*. Chorley (1948) subsequently showed, however, that the scent of a mixture of cattle dung and urine was attractive to *G. pallidipes*. Owaga (1984, 1985) later found urine of the African buffalo [*Syncerus caffer* (Sparrman)] to be a potent olfactory attractant for *G. pallidipes* and enhanced its potency by aging it for three weeks or longer.

The attraction of certain tsetse species, e.g., *G. pallidipes* and *G. morsitans* Westwood, to buffalo and cattle urine is attributable to their phenolic constituents. In the most attractive fraction of buffalo urine, Hassanali et al. (1986) identified seven phenols. Field subtraction bioassays revealed that the synergistic action of only two components (4-cresol and 3-*n*-propylphenol) was responsible for increased trap catches of *G. pallidipes* in Kenya (Owaga et al., 1988). In a similarly attractive fraction of cattle urine, Bursell et al. (1988) identified eight

phenols; the previously mentioned phenols (4-cresol and 3-*n*-propylphenol) were again essential for the fraction's attractiveness of *G. pallidipes* and *G. morsitans* in Zimbabwe (Vale et al., 1988). Of these two essential phenols, 3-*n*-propylphenol was critical for the attraction of tsetse to aged urine of buffalo and cattle (Owaga et al., 1988; Vale et al., 1988).

Tsetse locates stationary hosts through odor-regulated anemotaxis, with visual attraction becoming important at close range (Vale, 1974, 1977). Approximately 90% of both *G. pallidipes* and *G. morsitans* that come to a host are attracted by its odor (Vale, 1974; Vale and Hall, 1985). In nature, the extent of host-urine odor involvement in this process in not clear. Nevertheless, Brightwell et al. (1991) and Saini et al. (1993) have suggested that phenols from cattle urine mediate in tsetse host location behavior.

Most mammals, unless purposely confined, do not remain at the location where they have urinated and/or defecated. The latter activities usually occur after the animals have watered or while they are grazing. Some favorite tsetse hosts, e.g., the bushpig [*Potamochoerus porcus* (Linnaeus)], even use latrines, i.e., established dung piles to which they walk deliberately to defecate (Kingdon, 1982, p. 208). Furthermore, once deposited in the soil, urine is subject to microbial degradation. The implications of these for tsetse trapping technology, which targets host-seeking individuals, prompted us to evaluate the ecological relevance of mammalian urine in host location by tsetse. The tsetse catch of traps baited with urine of selected bovid hosts and a nonhost as well as the effect of soil on the attractiveness of cattle urine to tsetse were compared in typical tsetse-inhabited ecosystems. The phenolic constituents of urine from selected wild mammalian tsetse hosts and a nonhost also were compared in laboratory studies.

METHODS AND MATERIALS

Urine Sources. Wild mammalian hosts and nonhosts of tsetse were chosen from results of tsetse bloodmeal analyses published by Weitz (1963) and Snow et al. (1988). The choice was further narrowed by accessibility of the animal for urine collection. The hosts selected were the African buffalo, bushpig, warthog [*Phacochoerus aethiopicus* (Pallas)], and domestic cattle; the nonhost was waterbuck [*Kobus defessa* group (Ogilby)]. The buffalo, cattle, and waterbuck were in captivity and fed a zero-grazing diet of hay and concentrates. The bushpig and warthog free-ranged in the wild. Urine was collected either as these animals urinated naturally (buffalo, cattle, and waterbuck) or from their urinary bladders excised upon slaughter (bushpig and warthog). Subsequent handling of the urine depended on the requirements of the experiments (see below).

Field Sites. Field studies were done at the Olkeramatian Group Ranch at Nguruman (1°50′S, 36°05′E) in Rift Valley Province, and the Gongoni Forestry

Reserve at Gazi (4°25′S, 39°28′E) in Coast Province, Kenya. Olkeramatian Ranch is within a typical semiarid agropastoral ecosystem infested with *G. longipennis* Corti and *G. pallidipes*. The experimental sites were located along the thicketed fringes of the Ewaso Ngiro River, to the northwest of the tsetse suppression zone of Dransfield et al. (1990). Gongoni Forestry Reserve is within the rainforest zone of the south Kenya coast, whose salient ecological features Snow et al. (1988) described. Like most of the zone, the forestry reserve is infested with *G. austeni* Newstead, *G. brevipalpis* Newstead, and *G. pallidipes*. The experimental sites were in the interior biotope of the forest's southern portion, which adjoins the Msambweni Development Corporation Farm at Gazi. Both field sites are designated protected wildlife areas and each has an ample assortment of mammalian fauna.

Effect of Urine of Selected Hosts and Nonhost on Trap Catch of Tsetse. All experiments were carried out during the cool dry season (July–September) at Nguruman, using NG2G traps (Brightwell et al., 1991) baited with buffalo, cattle, or waterbuck urine aged for four weeks in the laboratory, following the procedure of Owaga (1985). The traps were placed at least 300 m apart.

The aged urine (200 ml) of each mammal was placed in a 250-ml glass jar dispenser with a mouth measuring 5.5 cm in diameter. Acetone (25 ml) was put into 200-ml glass dispensers, each of which had a 3-mm hole in its lid. The treatments consisted of traps baited with dispensers of urine and acetone placed beside each other at ground level, approximately 30 cm behind the trap entrance. The controls consisted of traps baited with acetone only.

Treatments and controls were deployed in a 4 × 4 Latin square replicated three times at two sublocations. There was a bait-free interval of four days between treatments. On the second day following positioning of odor baits, the traps were emptied to record the sex and number of each tsetse species caught.

Effect of Soil Substrate on Attractiveness of Cattle Urine to Tsetse. Experiments were conducted during the hot dry season (January–March) at the Gongoni Forestry Reserve using the NG2G trap. For this series of experiments, urine was obtained from Zebu cattle maintained under free grazing at Gazi. Large glass containers (one liter capacity, 15 cm deep) filled with soil dug up from Gongoni Forest or 250-ml glass jars without soil were used to dispense the urine odor. In the first experiment, 200 ml of fresh urine was poured into each dispenser and immediately used as bait for the traps. In the second experiment, the dispensers were left in the forest for three days after adding fresh urine before being used to bait traps from the fourth day. The treatment bait consisted of both urine and acetone; the control consisted of acetone alone.

Treatments were deployed in a 3 × 3 Latin square replicated three times at two sublocations that were ca. 500 m apart. The traps were emptied after 24 hr to record the sex and number of tsetse caught.

Extraction of Urine from Selected Hosts and Nonhost of Tsetse. Aged (3- to 6-week-old) urine samples (≈ 100 ml) of buffalo, bushpig, cattle, warthog, and waterbuck, respectively, were extracted twice with dichloromethane (20 ml × 2). The combined organic extract of each was dried over sodium sulfate, filtered, and concentrated in vacuo at 35°C. The residues were dissolved in dichloromethane (1 ml) and stored at −20°C.

Analysis of Urine Extracts. Urine extracts were analyzed on an HP 5890 gas chromatograph equipped with a flame ionization detector (FID) and an HP fused-silica capillary column (Carbowax 20 M, 50 m × 0.2 mm ID × 0.2 μm). The carrier gas was nitrogen at a flow rate of 0.62 ml/min. The GC oven temperature program consisted of an initial ramp of 100–180°C at 3°C/min, followed by a ramp from 180 to 230°C at 10°C/min. Chromatographic peaks were integrated on an HP 3393A integrator. The GC-MS analyses of urine extracts and synthetic standard compounds were conducted on a VG Masslab 12-250 mass spectrometer (EI mode, 70 eV; filament emission current 100 μA; source temperature 180°C; tuning standard PFTBA; GC interface temperature 220°C) coupled to an HP 5790 gas chromatograph under the same GC conditions as above.

Statistical Analysis. Trap catches were transformed to log $(n + 1)$ before being subjected to analysis of variance (ANOVA). If differences between the catch of female and male tsetse were negligible, the data for both sexes were pooled. When the treatment effect on trap catch was significant, differences between the treatment means were scrutinized for statistical significance at $P < 0.05$ by the Student-Newman-Keuls (SNK) test.

RESULTS

Effect of Host and Nonhost Urine on Trap Catch of Tsetse at Nguruman. Traps baited with the aged urine of buffalo, cattle, or waterbuck (treatments) caught significantly more *G. pallidipes* ($P < 0.001$) and *G. longipennis* ($P < 0.001$) than unbaited traps (controls). The source of the urine seemed to have affected the mean trap catch of *G. longipennis* but not that of *G. pallidipes* (Table 1). Nevertheless, the mean catch of either tsetse species in traps baited with the urine of buffalo and cattle (tsetse hosts) and waterbuck (a nonhost of tsetse) did not differ significantly.

Effect of Soil Substrate on Attractiveness of Cattle Urine to Tsetse in Gongoni Forest. Baiting traps with fresh cattle urine increased the catch of *G. pallidipes* and *G. brevipalpis* but tended to depress that of *G. austeni* (Table 2). The traps baited with fresh cattle urine in containers without soil caught only ~1.2 and ~1.3 times more *G. brevipalpis* and *G. pallidipes*, respectively, than those baited with fresh urine in soil-filled containers. Because of the low num-

TABLE 1. EFFECT OF AGED URINE OF SELECTED MAMMALS ON NG2G TRAP CATCH OF TSETSE AT NGURUMAN, RIFT VALLEY PROVINCE, KENYA[a]

Urine source	Catch/trap (mean ± SE)	
	Glossina longipennis	*Glossina pallidipes*
Buffalo	90 ± 18a	148 ± 34a
Cattle	52 ± 8a	120 ± 22a
Waterbuck	74 ± 11a	123 ± 33a
Control (no urine)	15 ± 4b	43 ± 12b

[a]ANOVA and SNK test for significant difference between treatment means were performed on log $(n + 1)$-transformed pooled trap-catch data. Means followed by the same letter are not significantly different ($P > 0.05$). Urine was aged for four weeks at the diurnal temperatures prevailing in the laboratory (22–25°C).

bers of *G. austeni* and *G. brevipalpis* caught, differences in their trap catch size were not subjected to statistical analysis. However, for *G. pallidipes*, whose catch sizes were statistically analyzable, the difference was not significant ($P > 0.05$).

The traps baited with cattle urine aged for three days in containers without soil caught almost twice as many *G. austeni*, *G. brevipalpis*, and *G. pallidipes* as those baited with urine aged in soil-filled containers. The latter did not attract significantly more tsetse than the unbaited control (Table 2). Again, low catch

TABLE 2. EFFECT OF AGING SUBSTRATE ON ATTRACTIVENESS OF CATTLE URINE TO TSETSE AT GAZI, COAST PROVINCE, KENYA[a]

Urine type/aging substrate	Catch/trap (mean ± SE)		
	G. austeni	*G. brevipalpis*	*G. pallidipes*
Fresh urine in container			
Without soil	0.5 ± 0.2	3.4 ± 1.0	27.1 ± 8.4
Filled with soil	1.0 ± 0.3	2.7 ± 1.0	20.4 ± 4.9
Control (acetone only)	1.2 ± 0.4	2.4 ± 0.5	18.3 ± 3.8
Urine aged in container			
Without soil	1.9 ± 0.7	2.5 ± 0.4	45.6 ± 4.2a
Filled with soil	1.0 ± 0.3	1.4 ± 0.4	19.2 ± 5.1b
Control (acetone only)	1.0 ± 0.5	2.3 ± 0.6	20.3 ± 7.7b

[a]ANOVA and, where applicable, SNK test for significant different between treatment means were performed on log $(n + 1)$-transformed pooled trap-catch data. Means in each column are not significantly different ($P > 0.05$) if followed by the same or no letter. The aging of urine lasted three days at diurnal temperatures inside Gongoni Forest (24–34°C).

excluded *G. austeni* and *G. brevipalpis* from statistical evaluation of these differences. However, for *G. pallidipes*, the substrate in which the cattle urine was aged had a statistically significant effect on the trap catch ($P < 0.01$). The urine was significantly more attractive when aged in a container without soil ($P < 0.05$).

Phenolic Constituents of Urine from Selected Hosts and a Nonhost of Tsetse. Aged urine of waterbuck contained both 4-cresol and 3-*n*-propylphenol (Figure 1A) in about the same ratio (~ 11 : 1) as that of buffalo (Figure 1B). The presence of 4-cresol and 3-*n*-propylphenol was confirmed by GC-MS analysis: 4-cresol, *m/z* (relative intensity) at 108 (86), 107 (100), 90 (12), 79 (25), 77 (80), 63 (10), 53 (15), 51 (20), 39 (18), 27 (20); 3-*n*-propylphenol, *m/z* (relative intensity) at 136 (39), 121 (15), 108 (52), 107 (100), 91 (8), 77 (30), 65 (7), 51

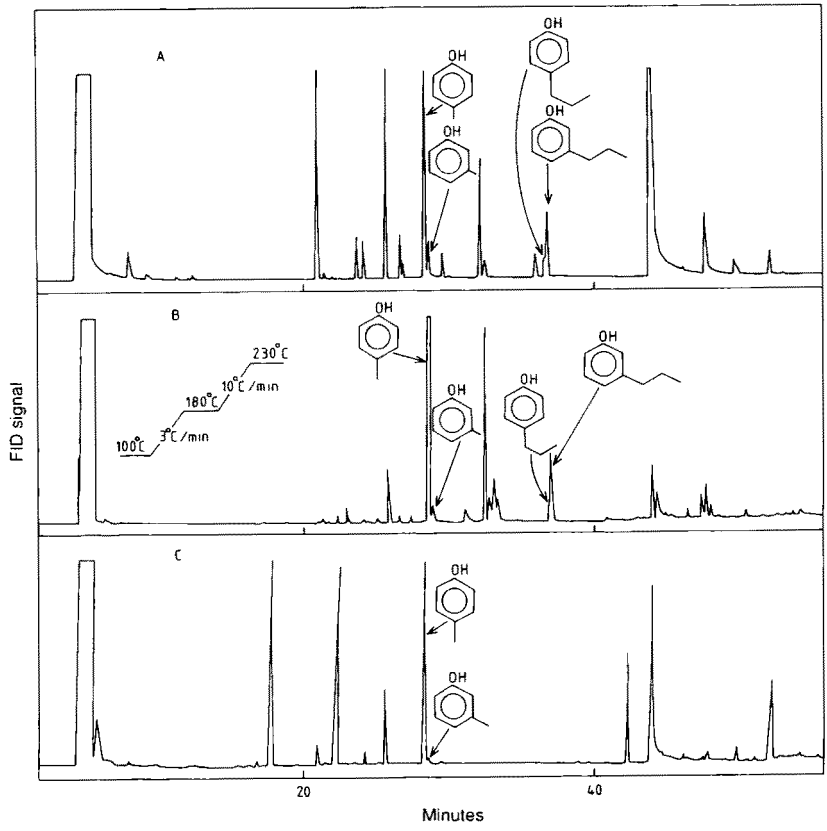

FIG. 1. Gas chromatograms of dichloromethane extracts of aged urine samples of the waterbuck (A), African buffalo (B), and warthog (C) showing the presence of 4-cresol in all the three but 3-*n*-propylphenol in only (A) and (B).

(9), 39 (18). These were identical to those of authentic synthetic standards (see also Hassanali et al., 1986). Aged urine of the warthog also contained 4-cresol but no observable amounts of 3-*n*-propylphenol (Figure 1C). Bushpig urine contained 4-cresol also, although in smaller amounts; in this case, 3-*n*-propylphenol was not observed.

DISCUSSION

The number of tsetse attracted by an odor-baited trap depends on the trap's collective olfactory and visual attractiveness to various tsetse species. The proportion of attracted flies caught depends on the trap's ensnaring efficiency. An attractive odor, applied as bait, would primarily enhance the number of tsetse attracted to the trap. By using tsetse traps of the same design for treatment and control, variability in trap catch due to the inherent efficiency of the trap design is reduced. The size of a baited trap's catch should reflect, therefore, the potency of the candidate tsetse attractant.

Aged urine of buffalo, cattle, and waterbuck, significantly increased the NG2G trap catch of the two tsetse species at Nguruman. The aged urine of tested hosts attracted *G. pallidipes* at about the same level. Aged cattle urine was apparently less attractive to *G. longipennis* than aged urine of either the buffalo or waterbuck. The chemoecological basis of these differences in the response of *G. pallidipes* and *G. longipennis* is not immediately clear, given the remarkable similarity in the phenolic composition of aged urine from all three bovids. Earlier studies, also at Nguruman, found similar differences in the effect of buffalo and cattle urine on trap catch to be statistically significant for *G. pallidipes* (Owaga, 1985) but not significant for *G. pallidipes* (Dransfield et al., 1986) and *G. longipennis* (Kyorku et al., 1990). Nevertheless, in our study, neither tsetse species showed evidence of having distinguished between traps baited with the urine of buffalo and cattle (hosts) and waterbuck (a nonhost). It is doubtful, therefore, that either species differentiates between these animals in nature solely from the scent of their aged urine.

The level of increase in the trap catch of *G. pallidipes* ($\times 1.8$) realized with fresh cattle urine in the Gongoni forest was relatively lower than that achieved with aged cattle urine at the same location ($\times 2.4$) or at Nguruman ($\times 2.8$). Significantly, according to Okech and Hassanali (1990), fresh buffalo urine has virtually no phenolic components. This is probably also true of fresh cattle urine. Since the attractiveness of aged buffalo or cattle urine to *G. pallidipes* depends on their phenolic constituents, the observed relative unattractiveness of fresh cattle urine is consistent with its probable chemical profile. It is thus improbable that urine attracts tsetse as wild animals pass it out, let alone attracting the flies to their hosts' locations.

The microbial invasion of fresh buffalo urine stored in an unsterilized container at room temperature for 16 days caused the formation and gradual accumulation of the phenols that attract tsetse (Okech and Hassanali, 1990). This study confirms that conversion of containerized fresh cattle urine into a tsetse attractant happens in as short an interval as three days under field conditions. It further shows that the substrate in which urine is aged greatly affects its transformation into a potent tsetse attractant. Thus, only when aged in a glass container, without soil, did the odor of cattle urine become sufficiently attractive to cause overall increases in the trap catch of tsetse. The outcome of aging urine in glass containers without soil is of debatable ecological relevance since, in nature, animals urinate on the ground.

A major advantage that chemical signals have over visual and auditory ones is that they can persist for some time without their sender being present (Eisenberg and Kleiman, 1972). In this study, however, cattle urine aged in soil-filled containers failed to increase significantly the trap catch of even *G. pallidipes*. This suggests that, under natural conditions, if the urine phenols essential for attraction of *G. pallidipes* happen to be produced, they do not persist. Therefore, cattle urine is an unlikely host-habitat chemical cue for this and probably other tsetse species.

By deterring microbial invasion of fresh urine through sterilization, Okech and Hassanali (1990) prevented buffalo urine from becoming attractive to tsetse. Although rich in microbial fauna and flora, soil may lack microbes that can hydrolyze the glucuronates and sulfates in fresh urine into the free phenols that make fermented bovid urine attractive to tsetse. Alternatively, the soil microflora may comprise organisms that produce these phenols and others that break them down rapidly as they are formed. Regardless of the mechanism, however, patches of soil wetted with fresh urine of cattle failed to produce significant increases in the mean trap catch of tsetse. Therefore, urine passed by moving animals is not likely to attract host-seeking tsetse to their hosts' immediate or subsequent locations.

Urinating on a social partner's body or transferring urine from one part of an animal's body to another is normal in the olfactory communication behavior of some mammalian species (Steiniger, 1950; Müller-Schwarze, 1971). In addition, the sweat glands of some mammals excrete, onto the skin surface, chemicals that are also found in their urine (Breazile, 1971). The action of microbial flora of the skin, or both urine and sweat, contributes to the total kairomonal mixture that gives an animal its body odor. For cattle, this mixture comprises all the phenols found in aged urine of buffalo or cattle and several other compounds (Saini et al., 1993). The latter are suspected of being more important to tsetse for host location and identification than the urine-derived phenols. The influence of these additional compounds may explain why the sebum of cattle significantly increased trap catches of *G. m. morsitans* (Warnes, 1990), whereas

aged urine of cattle did not (Vale et al., 1986, 1988). For the bushpig and warthog, however, it is unlikely that 3-*n*-propylphenol from urine or sweat will be among the kairomonal constituents of body odor. Bursell et al. (1988) did not find 3-*n*-propylphenol in aged domestic pig urine; neither was it found in the aged urine of the bushpig or the warthog in the present study. Therefore, pigskin wash should contain no more than trace amounts of the 3-*n*-propylphenol, which is critical for the attractiveness of aged mammalian urine to *G. pallidipes* (Owaga et al., 1988; Vale et al., 1988). Nevertheless, pigskin extract reportedly doubled and even quintupled the catch of *G. pallidipes* (Vanderplank, 1944; Persoons, 1965, 1966, 1967), suggesting that other volatile components are involved in the attraction of this tsetse. Accordingly, the presence of urine as a contaminant on the mammalian skin might be of little or no significance in attracting tsetse to hosts.

Cattle and buffalo, whose aged urine increased the trap catch of all the tsetse species in our study, are acknowledged hosts of many tsetse species (Weitz, 1963). However, *G. pallidipes* and *G. morsitans* are indifferent to the scent of aged urine of the warthog (Owaga, 1984) and the domestic pig (Bursell et al., 1988), which are also hosts of both tsetse species. In fact, Torr (1994) classified the urine of warthog as repellent to tsetse. These results seem to reflect the absence of biologically significant amounts of 3-*n*-propylphenol in aged urine of some favorite hosts of the tsetse (e.g., the domestic pig, bushpig and warthog). The scent of urine is thus of doubtful ecological relevance in the location and selection of hosts by tsetse.

In this study, *G. pallidipes* was attracted equally to the aged urine of waterbuck (a nonhost) and buffalo (a favorite host) in the field. Positive response by this tsetse species to the odor of fermented mammalian urine, therefore, does not necessarily signify a response to olfactory stimuli from a host. Perhaps this is why traps baited with aged urine of cattle caught fewer *G. pallidipes* that were foraging for a bloodmeal than those that were not (Madubunyi, 1995). A successful bout of host-seeking by tsetse ends in bloodsucking, during which it may pick up and/or transmit trypanosomes, an epidemiologically critical behavior common to all species of tsetse. It makes them all equally vulnerable to trapping and makes odor-baited traps potentially powerful tools for reducing trypanosomiasis transmission. Results obtained during this study suggest that achieving this goal would require odor-baits from a source other than mammalian urine.

Adsorbent-trapped skin volatiles of different mammalian hosts of tsetse contain a rich blend of chemicals (Hassanali, unpublished). These may include those kairomones suspected to be more potent than cattle urine phenols for host location (Saini et al., 1993) or the attraction of tsetse that are strictly foraging for a bloodmeal (Madubunyi, 1995). Skin-based constituents of body odor are chiefly secretions and exudations of glands that are part and parcel of mammalian dermatography. These are more likely than urine to possess species- or strain-

specific qualities by means of which a tsetse can make olfactory distinctions between its hosts and nonhosts or between potential hosts. The skin of their mammalian hosts is thus a promising source to prospect for kairomones that are critical for the location of hosts by each tsetse species.

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INTRAPOPULATIONAL CHANGES IN SEX PHEROMONE COMPOSITION DURING SCOTOPHASE IN ORIENTAL TOBACCO BUDWORM, *Helicoverpa assulta* (GUENÉE) (LEPIDOPTERA: NOCTUIDAE)

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Abstract—Solvent extracts of individual pheromone glands were prepared from female *Helicoverpa assulta* (Guenée) at 2-hr intervals throughout the scotophase. The amounts of female sex pheromone components, (Z)-9-hexadecenal, (Z)-11-hexadecenal, (Z)-9-hexadecenyl acetate, and (Z)-11-hexadecenyl acetate, in the extracts were determined by gas chromatographic analysis. Although females called from early scotophase (2 hr) until late scotophase (6 hr) the quantity of extracted pheromone remained high at 8 hr, the end of the scotophase. More than 70% of the pheromone gland extracts contained sex pheromone components regardless of whether the donor females had been called or resting. Pheromone components were absent from gland extracts prepared at the onset of the scotophase. The quantity of (Z)-9-hexadecenal and (Z)-11-hexadecenal increased rapidly to reach a maximum of approximately 260 and 30 ng/female, respectively, that was maintained for up to 8 hr, the duration of the scotophase. The quantity of (Z)-9-hexadecenyl acetate and (Z)-11-hexadecenyl acetate increased continuously during the scotophase to peak at 600 and 30 ng/female, respectively, 8 hr into the scotophase. At the end of scotophase the quantity of all pheromone components decreased significantly.

Key Words—*Helicoverpa assulta*, Noctuidae, Lepidoptera, calling behavior, sex pheromone composition, (Z)-9-hexadecenal, (Z)-11-hexadecenal, (Z)-9-hexadecenyl acetate, (Z)-11-hexadecenyl acetate.

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INTRODUCTION

Oriental tobacco budworm, *Helicoverpa assulta* (Guenée) (Lepidoptera: Noctuidae), is widely distributed throughout Asia, Oceania, and Africa. Sugie et al. (1991) identified hexadecanal, (Z)-9-hexadecenal (Z9-16:Al), (Z)-11-hexadecenal (Z11-16:Al) and (Z)-9-hexadecenyl acetate (Z9-16:Ac) as female sex pheromone components of *H. assulta* in Japan. However, mixtures of Z9-16:Al, Z11-16:Al, Z9-16:Ac, and (Z)-11-hexadecenyl acetate (Z11-16:Ac) caught the highest number of males in Korea, China, and Thailand, although the optimal ratio of compounds varied by country. Such intraspecific variation has been reported from a number of species (Arn et al., 1983; Haynes and Hunt, 1990; Löfstedt, 1990; Clearwater et al., 1991; Tòth et al., 1992), and it has been suggested that pheromone component ratio is genetically controlled in some lepidopteran species (Collins and Cardé, 1985; Haynes and Hunt, 1990; Hunt and Haynes, 1990). In contrast, the ratio of two sex pheromone components of *Phthorimaea operculella* was influenced by rearing temperature (Ono, 1993).

The two hexadecenyl acetates, Z9-16:Ac and Z11-16:Ac, were also found to elicit significantly different behavioral responses from male *H. assulta* in Korea (Cork et al., 1992; Park et al., 1994), China, Thailand (Cork et al., 1992), and Japan (Sugie et al., 1991). In Korea and Japan the hexadecenyl acetate isomers increased trap catches, while in China they both increased and decreased trap catch depending on season; in Thailand they had no significant effect on male trap catches.

Sex pheromone production and release in moths is dependent on both exogenous (photoperiod, temperature) and endogenous (neural, hormonal) factors (Baker and Cardé, 1979; Raina, 1988; Kou, 1992). The calling and mating activities of *H. assulta* were found (Kim and Boo, 1986; Cho and Boo, 1988) to have a circadian periodicity, with a peak in mating activity between 1 and 2 hr into the scotophase. Many Lepidoptera exhibit a similar diel periodicity in calling (McNeil, 1991; Giebultowicz et al., 1992) that has frequently been related to changes in pheromone titer, as reported for *H. zea* (Boddie) (Raina et al., 1986), *Pseudaletia unipunctata* (Haworth) (Delisle and McNeil, 1987), *Spodoptera littoralis* (Boisduval) (Martinez and Camps, 1988), *Spodoptera frugiperda* (J. E. Smith) (Ramaswamy et al., 1988), *Helicoverpa armigera* (Hübner) (Rafaeli and Soroker, 1989), *Heliothis virescens* (F.) (Mbata and Ramaswamy, 1990), *P. operculella* (Zeller) (Ono et al., 1990), and *H. assulta* (Kamimura and Tatsuki, 1994).

The effectiveness of sex pheromones for monitoring or control of insect pests may depend on the relative attractiveness of the synthetic sex pheromone compared to that of naturally produced pheromone. Therefore, an understanding of the temporal and spatial variability in sex pheromone composition of a species may have significant implications for a pheromone-based pest management strat-

egy. This study was conducted to investigate the periodicity of sex pheromone biosynthesis and composition in a population of the oriental tobacco budworm.

METHODS AND MATERIALS

Insects. Larvae of *H. assulta* were collected from hot pepper fields in Suwon, Korea, and reared individually on an artificial diet (Park, 1991). The colony was maintained at 25 ± 1°C for successive generations under a light–dark cycle of 16L:8D. Pupae were collected daily, sexed, and kept individually in Perspex containers (5 cm high × 3 cm diam.) under the same environmental conditions.

Observation of Calling Behavior and Pheromone Extraction. Adult virgin female *H. assulta*, 1–2 days old, were placed individually in plastic containers ($N = 37$) 2 hr before the onset of the scotophase. Two-minute observations were made of each female during each ensuing 2-h interval until 2 hr after the scotophase. Females were classified as either calling or resting. Calling females extruded their abdominal tips and displayed wing fanning over the entire period of observation, while resting females displayed negligible movement with wings and antennae folded (Cho and Boo, 1988).

Pheromone extracts were prepared at the end of each 2-hr observation period from virgin females classified as either calling or resting. Pheromone glands were excised by anesthetizing adult females with CO_2 and pressing the abdominal tip lightly to evert the ovipositor and the pheromone gland, a complete ring of glandular epithelium between the 8th and 9th abdominal segments (Moon et al., 1986). The gland and exposed abdominal tip were excised with microscissors into a microvial containing 10 μl hexane and 100 ng (*Z*)-9-tetradecenyl acetate as an internal standard, held for 30 sec, and then removed. The effect of prolonged extraction was tested by removing a 5-μl aliquot from the extract 30 sec after immersing the abdominal tip, adding a further 10 μl of hexane, without internal standard, to the vial, and taking a second extract after 300 sec had elapsed. Extracts were transferred to 10 μl glass capillary tubes, sealed, and stored at < -20°C.

Gas Chromatography (GC). Individual gland extracts were analyzed on a polar fused-silica capillary column (CBP-20, 25 m × 0.2 mm ID) in a Shimadzu model 14A GC equipped with a Grob split–splitless injector (210°C) and a flame ionization detector (210°C) with nitrogen carrier gas (2.0 kg/cm^2). Analyses were conducted with 1-μl aliquots injected onto the column held isothermally at 180°C.

Statistical Analysis. The results were statistically analysed by chi-square tests, Student's *t* test, or analysis of variance (ANOVA). If significant differences were found by ANOVA ($P = 0.05$), the means were ranked by Duncan's multiple-range test (DMRT).

RESULTS

Female *H. assulta* showed typical calling behavior (Cho and Boo, 1988) only during the scotophase. High incidence of calling behavior (80%) occurred within 2 hr of the onset of the scotophase and lasted up to 6 hr into the scotophase at essentially the same level (Figure 1). Two hours before the end of the scotophase, the level of calling decreased to about 30%, and none was observed in the photophase. Insects that were not calling showed typical resting behavior.

The percentages of gland extracts produced from calling and resting females during the scotophase that contained sex pheromone components were not significantly different (κ^2 test, $P > 0.05$), at 71 and 80%, respectively. Sixty-seven percent of resting females examined 2 hr after the end of the scotophase contained detectable amounts (>5 ng) of sex pheromone components. No sex pheromone components (<5 ng) were detected from gland extracts prepared within 2 hr of the onset of the next scotophase.

The quantity of the two major components of the female sex pheromone, Z9-16:Al and Z11-16:Al, reached an average of 189 and 23 ng/female, respectively, within 2 hr after the onset of the scotophase; thereafter the quantities remained statistically the same (Table 1). Two hours after the scotophase ended,

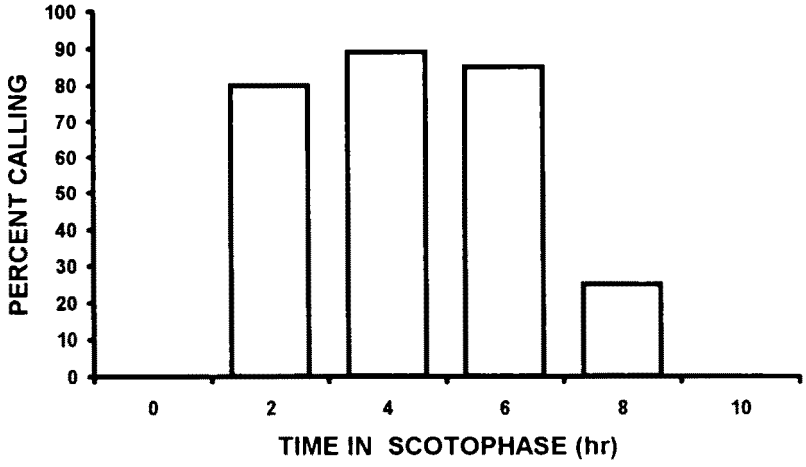

FIG. 1. The percentage of Korean female *H. assulta* calling during the scotophase under 16L:8D photoperiod at 25°C. Observations commenced 2 hr before the onset of the scotophase and terminated 2 hr after the end of the scotophase. Each female was maintained individually in a transparent polystyrene container ($N = 37$); 0 hr means 5 min before the onset, and 8 hr means 5 min before the end of scotophase. No calling was observed 2 hr before the onset of scotophase.

TABLE 1. AMOUNT OF EACH PHEROMONE COMPONENT IN FEMALE *H. assulta* GLAND EXTRACTS EXTRACTED BEFORE, DURING, AND AFTER SCOTOPHASE

Sampling time	Amount (ng) of extracted compound (average ± SE, $N = 8$)[a]			
	Z9-16:Al	Z11-16:Al	Z9-16:Ac	Z11-16:Ac
5 min before onset of scotophase	0a	0a	0a	0a
Within scotophase				
2 hr	189 ± 125bc	23 ± 14b	70 ± 70b	6 ± 6b
4 hr	260 ± 79c	30 ± 15b	122 ± 48b	9 ± 5b
6 hr	238 ± 108bc	27 ± 18b	344 ± 77c	25 ± 12b
7 hr 55 min	261 ± 83c	21 ± 10b	614 ± 262c	32 ± 20b
2 hr after scotophase	57 ± 14b	4 ± 4a	143 ± 83b	6 ± 4b

[a]Data within a column analyzed by ANOVA; means followed by different letters are significantly different at $P < 0.05$ by Duncan's multiple-range test.

the amount of each hexadecenal isomer declined significantly to 22 and 19%, respectively, of the levels 5 min before the end of the scotophase.

In contrast, the two hexadecenyl acetate isomers, Z9-16:Ac and Z11-16:Ac, increased continuously in quantity during the scotophase, statistically significantly for Z9-16:Ac (Table 1). The quantities peaked at 614 and 32 ng/female for Z9-16:Ac and Z11-16:Ac, respectively, and rapidly decreased within 2 hr after the scotophase to reach 23 and 19% of the respective levels just before the end of the scotophase.

The ratios of the amount per female of Z9-16:Al to Z11-16:Al and that of Z9-16:Ac to Z11-16:Ac were relatively constant throughout the scotophase, and averaged 14.6 ± 1 and 17.2 ± 1.5, respectively. The ratio between the ratios (aldehyde-acetate) was also relatively constant, averaging 0.83 ± 0.05. However, the ratios of each hexadecenyl acetate isomer to the homologous hexadecenal isomer changed significantly during the scotophase, rising from 0.37 and 0.26 at 2 hr into the scotophase to 2.35 and 1.52 just before the end of the scotophase for Z9-16:Ac/Z9-16:Al and Z11-16:Ac/Z11-16:Al, respectively (Figure 2). These changes were primarily due to the increased amounts of the hexadecenyl acetate isomers observed over the course of the scotophase and not to a decrease in the quantity of hexadecenal isomers.

Increasing the extraction time from 30 and 300 sex had no effect on the amount of Z9-16:Al and Z11-16:Al extracted (t test, $P > 0.05$), but significantly increased the amounts of Z9-16:Ac and Z11-16:Ac extracted by 38.9 ($P < 0.01$) and 23.4% ($P < 0.05$), respectively.

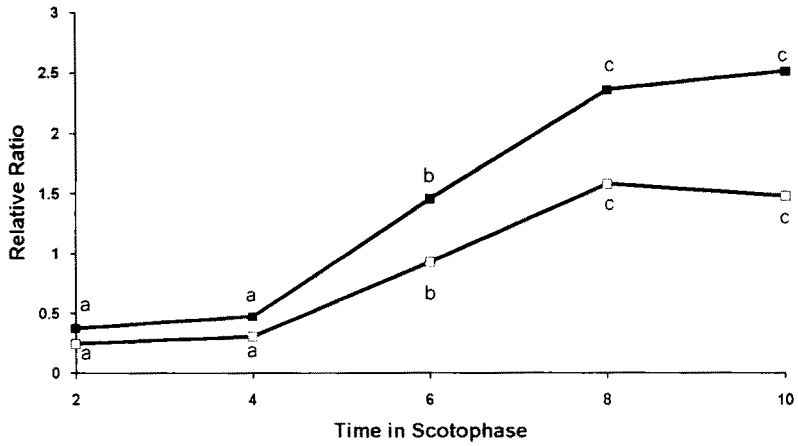

FIG. 2. Changes in the relative ratio of Z9- (solid square) and Z11-16:Ac (open square) to homologous hexadecenal isomers at different times in the scotophase in gland extracts of female *H. assulta* (16L:8D photoperiod). Values in a line with different letters are significantly different, $P < 0.05$.

DISCUSSION

The duration of calling observed in this study, from 2–6 hr into the scotophase (Figure 1), is similar to that found previously with insects of Korean (Kim and Boo, 1986; Cho and Boo, 1988) and Japanese origin (Kamimura and Tatsuki, 1993, 1994), although it contrasts with that observed for *H. assulta* from Thailand (E. Dunkelblum, personal communication), where calling was not observed until 10 h into the scotophase.

The continued high levels of extractable hexadecenal isomers (Table 1), even after calling activity declined (Figure 1), is in contrast to the findings of Kamimura and Tatsuki (1994), who found that the quantity of Z9-16:Al in gland extracts declined significantly as calling activity decreased. Since pheromone glands would be retracted when females are not calling, only enzymatic degradation could account for pheromone loss during this period. Because quantities of the hexadecenal isomers in the gland extracts did not decline during this period (Table 1), we propose that different mechanisms must control calling behavior and pheromone degradation. This hypothesis is supported by our finding that the amounts of pheromone components were independent of the behavioral status of female *H. assulta* throughout the scotophase (Table 1, Figure 2), a result that is consistent with the suggestion that calling and pheromone production are activated by different systems (Kamimura and Tatsuki, 1994).

The highly significant reduction in the pheromone content of gland extracts after the onset of the photophase (Table 1) suggests that the compounds were enzymatically degraded since loss through release would again be highly unlikely in the absence of calling. Whether this degradation is endogenously controlled by a circadian rhythm or is induced by exogenous stimuli, e.g., the presence of light, is unclear.

The absence of pheromone in gland extracts prepared 2 hr before the onset of the scotophase is in contrast to the results of Kamimura and Tatsuki (1993), who found that up to 20 ng/female of Z9–16:Al was present in Japanese *H. assulta* during the photophase.

The relative amounts of pheromone components in pheromone glands at different times through the scotophase is known to vary widely in the Heliothidinae. In *Helicoverpa zea* the quantity of pheromone components in gland extracts increased gradually after the onset of the scotophase to a maximum between 4 and 6 hr into the scotophase and then gradually declined (Heath et al., 1991). In contrast, pheromone content in *H. virescens* peaked between 8 and 10 hr into the scotophase (Heath et al., 1991). However, in both cases peak pheromone levels and calling activity coincided.

Cork et al. (1992) reported that the ratio of Z9–16:Ac to Z9–16:Al in pheromone gland extracts of female *H. assulta* from Korea increased dramatically between 4–6 and 10 hr into the scotophase from 0.26 to 2.32, respectively, in agreement with our finding of an increase of 0.37 to 2.35 between 2 and 8 hr into the scotophase. In contrast, Sugie et al. (1991) showed that the ratio of Z9–16:Ac to Z9–16:Al in Japanese females was 0.15 at the mid-scotophase. This result was subsequently confirmed by Kamimura and Tatsuki (1994), who also suggested that the relative amounts of each of the minor pheromone components remained constant despite daily fluctuations in the absolute amount of pheromone. In fact, their results do show a relative increase in the ratio of Z9–16:Ac to Z9–16:Al towards the end of the scotophase, but the change is more a reflection of a decrease in Z9–16:Al content rather than an increase in the amount of Z9–16:Ac. The reasons for the differences between the Korean and Japanese populations of *H. assulta* are unknown, but since a trap bait comprising a 3:1 ratio of the hexadecenal to hexadecenyl acetate isomers was more attractive than the hexadecenal isomers alone in Korea (Cork et al., 1992) the hexadecenyl acetates in Korea at least are behaviorally active. In related work, Heath et al. (1991) were able to show similar temporal changes in the relative proportions of alcohols to acetates and acetates to aldehydes in *Heliothis subflexa*, although in the related *H. virescens* these differences were not observed.

The increase in amounts of Z9–16:Ac and Z11–16:Ac recovered after a 10-fold increase in extraction time suggests at least some of the acetates were present inside the pheromone glands. The amounts of Z9–16:Al and Z11–16:Al recovered were not affected by extraction time, suggesting that they were only

present on the cuticular surface of the gland or are biosynthesized at or near the cuticular surface, as suggested by Teal and Tumlinson (1986) for the related *H. zea* and *H. virescens*. However, unlike *H. zea* and *H. virescens*, the presumed precursor alcohols could be synthesized from the corresponding hexadecenyl acetates that are present in the pheromone glands during the scotophase in significant quantities.

In conclusion we have shown that, in contrast to Japanese female *H. assulta*, Korean moths produce large quantities of the hexadecenyl acetate isomers, Z9–16:Ac and Z11–16:Ac, and that the relative ratios of the hexadecenal to hexadecenyl acetates vary significantly over the scotophase but are independent of the behavioral status of the females. These hexadecenyl acetates elicit a behavioral response from Korean *H. assulta*, but in China they had either no effect or reduced trap catches, depending on the time of year they were field tested (Cork et al., 1992). Thus their presence and bioactivity may define, in part, the basis for differences between geographically separated populations of *H. assulta*.

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IDENTIFICATION OF MAJOR COMPONENTS OF LARVIPOSITION PHEROMONE FROM LARVAE OF TSETSE FLIES *Glossina morsitans morsitans* WESTWOOD AND *Glossina morsitans centralis* MACHADO

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Abstract—The presence of pheromones produced by larvae of *Glossina morsitans morsitans* and *G. m. centralis*, respectively, which attract gravid females and result in aggregation of pupae, is confirmed. Behavioral experiments indicated that females preferred to larviposit over moist sand conditioned by previously allowing larvae to pupariate in it. Similar results were obtained with filter papers contaminated with the prepupariation excretions of larvae and with volatiles collected from larvae prior to pupariation. *n*-Pentadecane and *n*-dodecane were identified as the dominant electrophysiologically active components of the larviposition pheromones of *G. m. morsitans* and *G. m. centralis*, respectively, by GC-EAD and GC-MS analysis of the trapped larval volatiles. Both identified compounds were shown to significantly attract gravid females to larviposition sites in laboratory behavioral assays.

Key Words—*Glossina morsitans morsitans*, *Glossina morsitans centralis*, tsetse flies, Diptera, Glossinidae, larviposition, pheromone.

INTRODUCTION

Tsetse flies (Diptera: Glossinidae: *Glossina*) mature their offspring in utero, giving birth to mature larvae that burrow into the soil and pupariate. Just before pupariation, larvae release an anal exudate that coats the larval cuticle (King-

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horn, 1912; Lamborn, 1915; Fiske, 1920; Nash et al., 1976; Rowcliffe and Finlayson, 1981; Zdarek and Denlinger, 1991). In preliminary experiments undertaken by Leonard and Saini (1993), it was shown that semiochemicals from the prepupariation excretions of larvae of *G. morsitans morsitans* Westwood and *G. moristans centralis* Machado attract gravid females and result in aggregation of puparia.

In the present studies, in which we used new, larger, two-choice behavioral chambers, we reconfirmed that the anal exudate of larvae of *G. m. morsitans* and *G. m. centralis* contains pheromones that attract conspecific gravid females. Similar results were obtained with solvent extracts of larvae and with volatiles collected from larvae prior to pupariation. We report the identification of the major components of the larviposition pheromones of *G. m. morsitans* and *G. m. centralis*, respectively.

METHODS AND MATERIALS

Rearing

G. m. centralis and *G. m. morsitans* females were reared in the ICIPE rearing facility at 25°C ± 2°C, 70 ± 5% relative humidity, and a 12 L:12 D photocycle. Females were mated three days after eclosion and blood-fed on rabbits three times a week. Flies in the first cycle of parturition were used for all experiments.

Conditioning of Sand

Six cages, each containing 20 gravid female *G. m. centralis* or *G. m. morsitans*, were placed over a tray of sand previously extracted with solvents and heat sterilized at 100°C for 24 hr. At parturition, larvae burrowed through the mesh bottoms of the cages, dropped into the sand, and pupariated. Flies larviposited over a four-day period, after which puparia were removed by sifting, with a recovery of 80 pupae.

Behavioral Assays

Experiment 1. In previous experiments (Leonard and Saini, 1993) flies were held in standard tsetse rearing cages (19.5 × 7 × 6 cm), and in choice experiments the conditioned or control sand covered only 30% of the surface of the tsetse cages. Many larvae were deposited in the middle region. In the present work, new, larger, two-choice larviposition chambers 60 × 60 × 60 cm were constructed. Three sides of these chambers were covered with nylon mesh. The back was of metal and the front was covered with a removable glass door. One of the sides had a port from which flies easily could be introduced. The floor

was divided into two equal halves by disposable trays 59 × 59 × 1 cm each containing 750 g of sterilized or contaminated sand moistened with 75 ml of distilled water. The sand was conditioned by heat sterilization as above. A total of 305 *G. m. morsitans* and 268 *G. m. centralis* gravid females that were 18 days old were released in 13 replicates, and the number of pupae deposited in each tray counted at the end of each test, which ran for 96 hr. All behavioral assays were undertaken at 25°C ± 2°C, 60 ± 5% relative humidity, and a 12 L:12 D photocycle.

Experiment 2. In the next series of experiments, freshly deposited third instars were removed from the tsetse insectary and placed in four Petri dishes, each containing a 9-cm-diameter filter paper. Each dish contained 20 larvae. Some of the anal secretions produced by the larvae 1–2 hr prior to pupariation were absorbed by the filter paper in the Petri dishes, and these impregnated filter papers were subsequently used in bioassays. Four such impregnated papers were randomly placed in one tray of the large larviposition chambers and covered with 750 g of sterilized sand moistened with 75 ml of distilled water. The other tray, containing four similar plain filter papers covered with moistened sterilized sand, served as a control. A total of 335 gravid *G. m. morsitans* in nine replicates and 431 *G. m. centralis* in 12 replicates, all 18 days old, were offered the choice of control or larval-material-impregnated papers for larviposition, and the number of pupae deposited in each tray was counted at the end of each 96-hr testing period.

Experiment 3: Collection of Volatiles from Larvae. Volatiles were collected from freshly deposited third instars by placing them in a short glass tube. The inlet was plugged with activated glass wool (to adsorb contaminants from incoming air and to prevent larvae from escaping), while the outlet contained a short column of activated charcoal and another plug of glass wool as the adsorbent materials. Glass wool was activated by heating at 100°C for 24 hr. Filtered air was passed over 50 larvae for 2 hr at a constant flow rate of 15 ml/min. The adsorbents were then washed with 10 ml each of hexane and dichloromethane in succession, the extracts pooled, and the volume reduced to 100 μl by blowing a slow stream of filtered nitrogen. Collection of volatiles was repeated on four sets of larvae (50/set) in a similar fashion and all the trapped volatiles pooled. From the total volume collected, 160 μl of trapped volatiles (from 40 larvae) was kept for GC and GC-MS analysis. The remaining 240 μl was diluted with hexane to 2 ml and used for behavioral bioassays.

Behavioral Assays with Collected Volatiles

Four filter papers (9 cm diam.) were each impregnated with 250 μl of the volatile collection in hexane (equivalent to 20 larvae/filter paper), randomly placed in one tray of the larviposition chambers, and covered with 750 g of

moistened sterilized sand (moistened with 75 ml of distilled water). The control side had four filter papers, each impregnated with 250 μl of hexane and covered with 750 gm of moistened sterilized sand. Gravid 18-day-old flies, totaling 120 each of *G. m. morsitans* and *G. m. centralis* in four replicates, were offered the above choices and the number of pupae deposited in each tray counted at the end of each 96-hr testing period.

Analysis of Volatiles

Aliquots (2 μl) of the extracts were analyzed by gas chromatography (GC) and by gas chromatography–mass spectrometry (GC-MS). GC analyses were performed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with a flame ionization detector (FID), an HP capillary column (methyl silicone, 50 m × 0.2 mm ID × 0.32 μm film thickness) using nitrogen as the carrier gas at a flow rate of 0.8 ml/min. GC conditions were optimized to allow adequate resolution of all candidate electrophysiologically active peaks. For the analysis of *G. m. morsitans* larval volatiles, the oven temperature was initially isothermal at 60°C (5 min) and then programmed to 190°C at 3°C/min and finally to 240°C at 5°C/min; for that of *G. m. centralis*, after an isothermal period at 60°C (5 min), the oven temperature was programmed to 240°C at 5°C/min. Chromatographic peaks were integrated by an HP 3396 integrator. GC-MS analyses were carried out on a VG Masslab 12-250 mass spectrometer (EI, 70 eV) coupled to an HP 5740 series A gas chromatograph.

Coupled gas chromatography–electroantennography (GC-EAD) was performed employing the same GC conditions as above. The column effluent was split 1 : 1 into two 50-cm-long deactivated fused silica columns, one connected to the FID and the other to an antenna of the appropriate gravid female. A make-up gas (40 ml/min) was added just before the split point to accelerate the effluent through the deactivated columns. The effluents were driven from the chromatograph through a transfer line maintained at 150°C by a THC-3 temperature control unit (Syntech) and into a moistened airstream (4.0 ml/sec, 90% relative humidity, 25°C) and focused on the antennal preparation via a stainless steel tube (5 mm ID). The return time constant was set at 2 sec. FID and EAD signals were monitored synchronously with a program on a GC-EAD interface (Syntech) card installed in a PC (Harvard Professional Computer, American Megatrends Inc.). The antennae for EAD recordings were prepared as described earlier (Leonard and Saini, 1993; Saini and Hassanali, 1991).

Responses of Gravid Females to Major Components of Larviposition Pheromones

Responses of gravid *G. m. morsitans* and *G. m. centralis* to the major components of their respective larviposition pheromones were determined in

two-choice behavioral bioassays similar to those conducted with volatiles collected from larvae (Experiment 3). Four filter papers (9 cm diam.) were impregnated with 10, 20, 40, 80, 160, or 320 larval equivalents of *n*-pentadecane or *n*-dodecane (major component of *G. m. centralis* larviposition pheromone). The dilutions of the compounds were made with paraffin oil to effect a gradual release of the compounds. The treated filter papers were randomly placed in one tray of the larviposition chambers and covered with 750 g of sterilized sand moistened with 75 ml of distilled water. The control side had four filter papers impregnated with an equivalent amount of paraffin oil and covered with 750 g of moistened sterilized sand. The number of 18-day-old gravid females of *G. m. morsitans* or *G. m. centralis* released per dose and the number of replicates are indicated in Tables 2 and 3 below. The number of pupae deposited in each tray were counted at the end of each 96-hr testing period.

RESULTS

Choice Tests

Results of the behavioral bioassays in the two-choice behavior cages (experiments 1–3) clearly showed that gravid *G. m. centralis* and *G. m. morsitans* females preferred to larviposit in sand conditioned with previous larvipositions (Table 1). Similar preference was shown by gravid females to filter papers contaminated with anal secretions and by volatiles collected from larvae prior

TABLE 1. LARVIPOSITION OF *G. m. morsitans* (Gmm) AND *G. m. centralis* (Gmc) GRAVID FEMALES WITH RESPECT TO VARIOUS TREATMENTS

Treatments	Species	Replicates (*N*)	Flies released (*N*)	Flies larviposited (*N*)	Response (%)	
					Control	Treate
Expt. 1						
Sand conditioned with larvae	Gmm	13	305	173	35.8b[a]	64.2a
vs. sterilized sand (control)	Gmc	13	268	124	38.7b	61.3a
Expt. 2						
Filter papers + anal secretions	Gmm	9	335	186	44.6b	55.4a
vs. clean filter paper (control)	Gmc	12	431	246	42.3b	57.7a
Expt. 3						
Sand + larval volatiles vs.	Gmm	4	120	52	25.0b	75.0a
sterilized sand (control)	Gmc	4	120	66	16.6b	83.4a

[a]Different letters in rows denote significant different ($P < 0.05$ student t test).

to pupariation (Table 1). Larval volatiles elicited the strongest larviposition response, 75% in the case of *G. m. morsitans* and 83% in *G. m. centralis*.

Analysis of Volatiles

Gas chromatographic analysis of trapped volatiles from larvae of the two tsetse species were different qualitatively and quantitatively (Figures 1 and 2). Repeated GC-EAD analyses on separate collections from *G. m. morsitans* consistently gave an EAD signal corresponding to a major peak and four weaker signals corresponding to blends of minor components (Figure 1). GC-MS analysis of the volatiles allowed the characterization of the major peak as *n*-pentadecane, *m/z* (rel. intensity) at 57 (100), 43 (90), 71 (65), 85 (45), 99 (18), 212 (18), 113 (6). This was confirmed by coinjection studies and analysis of authentic samples by GC-EAD. Similar analyses with collections from *G. m. centralis* (Figure 2) showed three sets of EAD signals, two corresponding to blends of minor components, and one to a major component identified as *n*-dodecane and confirmed by comparison (GC-EAD, GC) with an authentic sample, *m/z* (rel. intensity) 57 (100), 43 (98), 71 (60), 85 (40), 170 (15), 98 (10). In both sets of analyses, the minor EAD active components could not be clearly separated and located. Identification of these components will be done by micropreparation of blends corresponding to these regions followed by rechromatography under different sets of conditions.

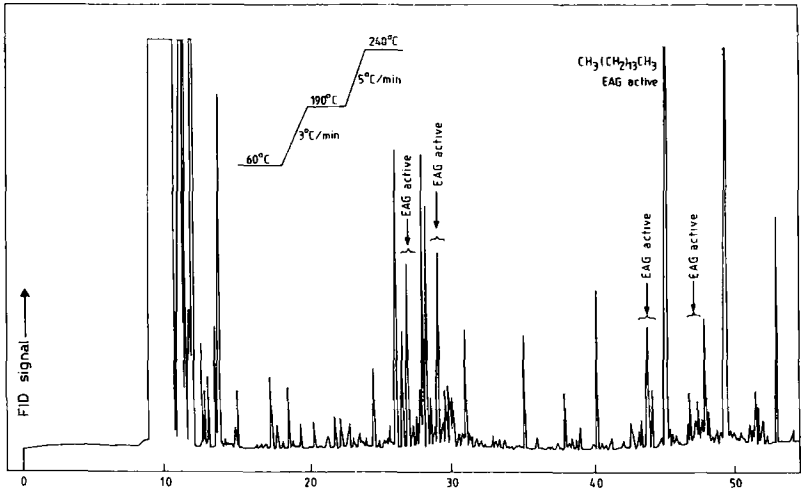

FIG. 1. Gas chromatogram of volatiles collected from larvae of *G. m. morsitans* and injected into a 50-m methyl silicone capillary column. Peaks and areas showing EAG activity in GC-EAD runs are indicated.

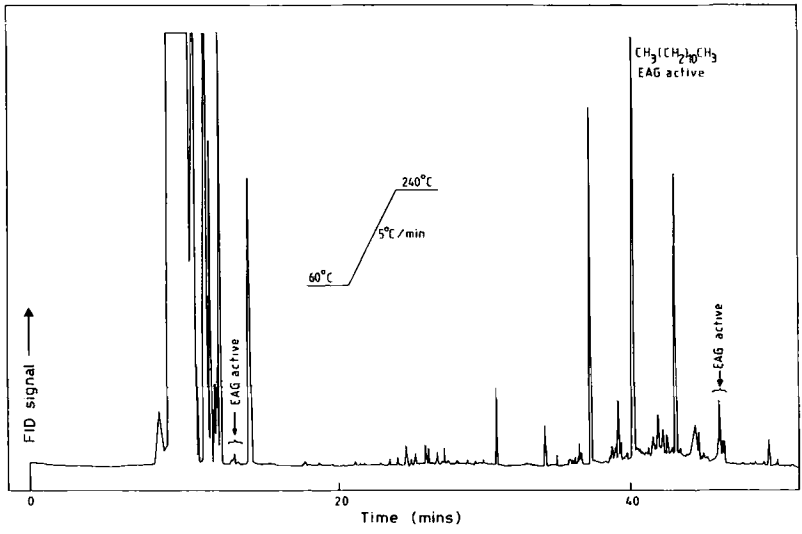

FIG. 2. Gas chromatogram of volatiles collected from larvae of *G. m. centralis* and injected into a 50-m methyl silicone capillary column. Peaks and areas showing EAG activity in GC-EAD runs are indicated.

Larviposition Responses of Gravid G. m. morsitans *and* G. m. centralis *to* n-*Pentadecane and* n-*Dodecane*

Tables 2 and 3 show the responses of flies to the major components identified from the larval volatiles as compared to the control. In the case of *G. m. morsitans* (Table 2), 60–80% of larvipositions occurred in the trays impregnated with 96–769 μg of *n*-pentadecane. Responses at lower doses of *n*-pentadecane

TABLE 2. RESPONSE OF LARVIPOSITING *G. m. morsitans* TO VARIOUS DOSES OF *n*-PENTADECANE

Treatment	Dose (μg)[a]	Replicates (*N*)	Flies Released (*N*)	Flies Larvipositing (*N*)	Response (%) Control	Response (%) Treated
Sand + *n*-	24	5	76	30	49.3a	50.7a
pentadecane	48	5	78	49	42.1a	57.9a
vs sterilized	96	10	201	149	32.6a	67.4b
sand	192	5	74	54	24.5a	75.5b
(control)	384	4	81	51	27.2a	72.8b
	769	4	74	54	21.7a	78.3b

[a]The lowest dose represents *n*-pentadecane equivalent of 2-hr collection of volatiles from 10 larvae.

TABLE 3. RESPONSE OF LARVIPOSITING *G. m. centralis* TO VARIOUS DOSES OF *n*-DODECANE

Treatment	Dose (μg)[a]	Replicates (*N*)	Flies Released (*N*)	Flies Laripositing (*N*)	Response (%)	
					Control	Treated
Sand + *n*-	23.4	5	76	46	46.0a	54.0a
dodecane	46.8	5	99	72	40.3a	59.7a
vs	93.6	9	166	119	35.4a	64.6b
sterilized	187.2	4	55	45	34.3a	65.7b
sand	374.4	3	40	24	38.5a	61.5b
(control)	750.0	5	79	40	53.4a	46.6a

[a]The lowest dose represents *n*-dodecane equivalent of 2-hr volatile collection from 10 larvae.

were not significantly different from controls. *G. m. centralis* showed significant responses at 93.6-, 187.2-, and 374.4-μg doses of *n*-dodecane (Table 3). At lower doses (23.4 and 46.8 μg) and doses higher than 374.4 μg, the responses were not significantly different from controls.

DISCUSSION

Experiments undertaken by Leonard and Saini (1993) confirmed the results of Nash et al. (1976) that gravid female *G. m. morsitans* preferred to larviposit in sand in which larvae had pupariated previously. Results of the current experiments clearly show that filter papers contaminated with the anal exudate of larvae and, in particular, volatiles collected from larvae of *G. m. morsitans* and *G. m. centralis* prior to pupariation were attractive to respective gravid females and resulted in aggregation of corresponding puparia.

The major electrophysiologically active compounds in the larval volatiles of *G. m. morsitans* and *G. m. centralis* were identified as *n*-pentadecane and *n*-dodecane, respectively. Both these compounds caused selection by gravid females for larviposition comparable to the larval volatiles. However, the role(s) of the unidentified electrophysiologically active compounds remains to be elucidated. The dose–response results obtained for *G. m. centralis* pheromone, unlike the response of *G. m. morsitans*, showed that larviposition became less discriminatory at high doses (Table 3). We attribute this effect to higher volatility of *n*-dodecane (rather than due to an intrinsic loss of activity at high concentrations) than that of *n*-pentadecane. It may be that at high doses increasing amounts of volatile hydrocarbon diffused within the entire behavior bioassay arena, thus blurring differences between the treated and control sides.

The known sex pheromones of *Glossina* species are all saturated hydrocarbons associated with their cuticular waxes. The finding that the dominant behaviorally active components of larviposition pheromones of *G. m. morsitans* and *G. m. centralis* are also saturated hydrocarbons, albeit of lower molecular weights, suggests a general pattern for the selection of this class of compounds as signals in intraspecific communication by tsetse flies. Studies on the larviposition pheromones of other species will help to establish the validity of this generalization. Other insects that also use simple hydrocarbons in intraspecific communication include the astigmatid mite *Caloglyphus rodriguezi* (Mori et al., 1995), the red wood ant *Formica lugubris* (Walter et al., 1993), and the gregarious cockroach *Blaberus craniifer* (Brossut et al., 1974).

Since both the identified compounds are fairly volatile, they could attract gravid females from a distance to larviposition sites. At close range, the physical nature of the larviposition sites and the microclimatic conditions, such as humidity, may be important in the selection of suitable larviposition sites. In this regard, the previous work of Leonard and Saini (1993) suggests that gravid females are most responsive to larval semiochemicals when the differential between soil moisture and ambient humidity is large. Whether the larviposition pheromones function solely as a cue to aggregate gravid females or also as a stimulus for larviposition is still not clear. Further behavioral work is needed to fully understand the selection of larviposition sites before the larviposition pheromones can be exploited to either increase the efficiency of existing odor-baited traps and targets by attracting more gravid females or in concentrating gravid females and pupae in areas where pathogens or other biological control agents could cause high mortality.

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CHEMISTRY OF VENOM ALKALOIDS IN SOME *Solenopsis* (*Diplorhoptrum*) SPECIES FROM PUERTO RICO

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Abstract—A number of 15-carbon alkaloids have been identified in venom extracts of four Puerto Rican species of ants in the genus *Solenopsis* (*Diplorhoptrum*). Workers of a species from El Verde produced the *cis* and *trans* isomers of 2-methyl-6-nonylpiperidine with the latter isomer predominating. The same compounds were identified in queens of a species from Río Grande, but in this species no alkaloids were detected in worker extracts. Workers of a *Diplorhoptrum* species collected on Mona Island produced primarily a *trans*-2-methyl-6-(*Z*-4-nonenyl)piperidine, **3**, with smaller amounts of the *cis* isomer, whereas the major compound found in the queens of the same species on Mona Island was (5*Z*,9*Z*)-3-hexyl-5-methylindolizidine, identical with the alkaloid produced by queens of a species collected on Cabo Rojo. Surprisingly, workers of the Cabo Rojo species produced (5*Z*,9*Z*)- and (5*E*,9*E*)-3-butyl-5-propylindolizidine (**4** and **5**, respectively) reported earlier as the 223AB indolizidines from skins of dendrobatid frogs. The possible significance of the qualitative and quantitative differences in the venom alkaloids synthesized by queens and workers is discussed as is the possibility that ants containing such

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alkaloids may serve as a dietary source for the skin alkaloids used by certain frogs in chemical defense.

Key Words—Venom, alkaloids, *Solenopsis*, Puerto Rico.

INTRODUCTION

A variety of saturated-ring nitrogen heterocycles have been identified in the venoms of ant species in the myrmicine genera *Monomorium* and *Solenopsis* (Jones et al., 1982a,b). The 3,5-dialkylindolizidines are one of the earliest reported structural classes of these compounds (Ritter et al., 1973). When they have been found with other structural types of ant venom alkaloids, their concomitants have been 2,5-dialkylpyrrolidines and 3,5-dialkylpyrrolizidines in *Monomorium* species (Ritter and Persoons, 1975; Jones et al., 1990a) and 2,6-dialkylpiperidines in *Solenopsis* species (Jones et al., 1984). The 3,5-dialkylindolizidines are often found among the skin alkaloids of dendrobatid frogs and occasionally occur in other amphibians while 2,5-disubstituted pyrrolidines, 3,5-disubstituted pyrrolizidines, and 2,6-disubstituted piperidines have also been reported as amphibian skin alkaloids (Daly et al., 1993).

The genus *Solenopsis* includes many minute species that are currently placed in a "subgenus" *Diplorhoptrum*, a large, worldwide group (Bolton, 1987) to which the species of the present study belong. In addition, in the past some "subgenera" (e.g., *Diphlorhoptrum*) have been elevated to the status of a genus, but they are now usually recognized as a synonym of *Solenopsis* (Ettershank, 1966). Here we use the name *Diplorhoptrum* as a matter of convenience without implying that it is a valid taxonomic entity.

In this report, the alkaloidal venom components of four collections of *Diplorhoptrum* spp. from Puerto Rico are described. Three of these collections, in which different castes were obtained, show conspicuous differences between alkaloids found in workers and in queens. In these collections 3,5-dialkylindolizidines and/or 2,6-dialkylpiperidines were detected, and of the former, the major alkaloid from the workers of one collection was found to be the indolizidine **223AB** (**5**) (Figure 1 below), first reported in dendrobatid frogs (Spande et al., 1981). While a number of alkaloids previously detected in ants have been found in amphibians, this is the first report of an alkaloid previously detected in amphibians, now being found in ants.

METHODS AND MATERIALS

Chemical Analyses

Gas chromatographic analyses and mass spectra were obtained using an LKB-2091 GC-MS, a Finnigan ITMS, or a Finnigan ion-trap model 800, all

equipped with 25-m × 0.32-mm-ID HP-5 fused-silica columns. The first two instruments used Hewlett-Packard 5890 gas chromatographs programmed from 60 to 250°C at 10°/min. The model 800 ion trap was interfaced with a Varian gas chromatograph model 3400 programmed from 100 to 280°C at 10°/min after a 1-min delay. FT-IR spectra were obtained using a Hewlett-Packard model 5965B detector (narrow band, 4000–750 cm^{-1}, resolution 8 cm^{-1}) interfaced with a Hewlett-Packard 5890 gas chromatograph fitted also with an HP-5 column programmed 100–280°C at 10°C/min after a 1-min delay. Data were analyzed with the 59970 IRD ChemStation. FT-IR spectra of neat liquids were obtained using a Perkin-Elmer series 1600 FT-IR instrument. High-resolution mass spectrometry (HRMS) used a JEOL SX102 instrument fitted with a 15-m × 0.20-mm HP-5 column.

Ants. Ants were collected in the areas (A–D) indicated in Table 1 below, and extracts were immediately prepared in glass vials containing methylene chloride. Voucher specimens of all samples were deposited in the collection of the Los Angeles County Museum of Natural History, Los Angeles, California. Each vial represented a different colony, and extracts of whole bodies were analyzed directly and individually. While the milking of stingers has demonstrated the venom glands to be the source of alkaloids detected in ants of the genera *Solenopsis* (cf. Brand et al., 1972; MacConnell et al., 1971) and *Monomorium* (unpublished work of Blum and Jones), the small size of the Puerto Rican *Diplorhoptrum* ants made this impractical. The alkaloid components of the extracts can be distinguished easily from the lipid components by their mass

TABLE 1. ALKALOIDS IDENTIFIED FROM PUERTO RICAN *Solenopsis (Diplorhoptrum)* SPECIES

	Alkaloids					
Collection site	*cis*-**1**	*trans*-**1**	**2**	**3**	**4**	**5**
A. El Verde						
workers	+	+++++	–	–	–	–
B. Río Grande						
workers	–	–	–	–	–	–
queens	+++++	+	–	–	–	–
C. Mona Island						
workers	–	–	+++	–	–	–
queens	–	–	t[a]	+++	–	–
D. Cabo Rojo						
workers	–	–	–	–	++	+++
queens	–	–	–	+++	t	t

[a] t = trace detected.

spectra, which generally have odd-mass molecular ions and even-mass fragments (for alkaloids with one nitrogen).

Based on the similarity of their size with ants of the genus *Monomorium* (cf. Jones et al., 1982b), we estimate very roughly that each *Diplorhoptrum* worker released, at most, 0.1–1.0 μg alkaloid.

Hydrogenation of Extract C. A slow stream of hydrogen was bubbled through a small portion of this extract in methanol in the presence of PtO_2 for 2–3 min.

Synthesis of Piperidines ***2a*** *and* ***2b***

*2-[3-(6-Methyl-2-pyridyl)propyl]-1,3-dioxolane (**6**).* A solution of 2,6-lutidine anion obtained from 6.4 g (60 mmol) of lutidine in 100 ml of diethyl ether and 38 ml of 1.6 M *n*-butyllithium in hexanes at 0°C was treated with a solution containing 7 ml of 2-(2-bromoethyl)-1,3-dioxolane in 30 ml of diethyl ether. After 0.5 hr, the mixture was poured into water and extracted with ether. The ether solution was dried over anhydrous K_2CO_3, and after filtration, the more volatile components were removed in vacuo to provide 9 g of an oil that was greater than 80% compound **6** by GC. IR data (neat) were 3054, 2948, 2874, 2769, 1590, 1574, 1457, 1410, 1373, 1135, 1097, 1034, 939, 817, 790 cm^{-1}; GC retention time was 9.1 min; MS *m/z* (relative intensity, percent, Finnigan model 800 ion trap mass detector): 208 (M + 1, 10), 162(13), 134(18), 107(100), 73(38). Compound **6** was accompanied by ca. 10% of a material of retention time 17.7 min; MS *m/z* 308 (M + 1, 100), 164(22), 162(17), 147(22), 134(15), 132(10), 107(22), 99(10), 73(32), which very likely was a bis-substituted lutidine. HRMS, calculated for $C_{12}H_{17}NO_2$: 207.1255; observed, 207.1258.

*2-Methyl-6-(Z,4-nonenyl)pyridine (**7**).* A mixture containing 6 g of dioxolane **6,** 50 ml of acetone, 50 ml of methanol, and 100 ml of water was carefully acidified with concentrated HCl to ca. pH 2 and stirred for 1.5 hr at room temperature. The mixture was neutralized with solid $NaHCO_3$, and most of the solvents were removed in vacuo. The mixture was extracted with diethyl ether, and the ether extracts were dried over anhydrous $MgSO_4$. Distillation provided 3.5 g of 4-(6-methyl-2-pyridyl)butanal, bp 64–67° (0.05 mm Hg); IR(neat) 3062, 2927, 2823, 2719, 1721, 1590, 1575, 1456, 791 cm^{-1}; GC retention time 5.6 min; MS *m/z* 164 (M + 1, 65), 146(3), 134(13), 120(5), 107(100), 65(8). HRMS, calculated for $C_{10}H_{13}NO$: 163.0094; observed, 163.0098. A mixture of 2.5 g (6 mmol) of *n*-pentyltriphenylphosphonium bromide in 25 ml of THF was cooled to 0°C and treated with 2.4 ml of 2.5 M *n*-butyllithium in hexanes. After 3 hr, a solution containing 1 g (6 mmol) of 4-(6-methyl-2-pyridyl)butanal in 5 ml of THF was added, and the mixture was stirred overnight. Following the addition of 50 ml of hexane and 25 ml of diethyl ether, the mixture was filtered through a Florisil pad and the solvent was removed in

vacuo. Redissolution and filtration was repeated to provide 1.25 g of crude **7** that was nearly 90% pure by GC analysis. IR(neat) 3067, 3005, 2943, 2923, 2861, 1656(w), 1591, 1578, 1456, 790 cm^{-1}; GC retention time 13.9 min; MS *m/z* 218 (M + 1, 12), 202(8), 132(8), 120(10), 107(100). HRMS, calculated for $C_{15}H_{23}N$: 217.1825; observed, 217.1819.

2-Methyl-6-(Z,4-nonenyl)piperidine (2). The procedure of Jones et al. (1990b) was applied to the preparation of **2.** A small portion (about 20 mg) of the Wittig product **7** in 1.0 ml of absolute ethanol in a 3-ml test tube was heated to 95°C in an oil bath and small chips of sodium metal added over a period of approximately 1 hr, adding fresh ethanol as necessary. On cooling to room temperature, the resulting solid was allowed to stand capped overnight at room temperature; then water was added and the solution stirred in a hot water bath under a stream of nitrogen to remove excess ethanol. The aqueous layer was extracted four times with small portions of ethyl acetate. These extracts were combined, dried (Na_2SO_4), and the solvent was evaporated. The residue was dissolved in 2.0 ml ethanol, the pH adjusted to 6 (checked with narrow range pH paper) with 10–15 drops of 0.5 N HCl, and a small portion (about 5 mg) of $NaCNBH_3$ (Aldrich) was added, followed by another, 1 hr later, as the originally turbid solution became clear. A third portion of $NaCNBH_3$ was added 2 hr later. The solution was stirred for 18 hr and then acidified with 0.5 N HCl until the evolution of gas ceased. The solution was evaporated under a nitrogen stream, made alkaline with 2 N KOH, and extracted four times with small portions of ethyl acetate. After drying, GC-MS showed two closely eluting peaks at 9.0 min and 9.4 min (3.2 : 1.0, respectively) with the same mass and infrared spectra as reported for the isomeric mixture **2a** and **2b,** respectively. HRMS calculated for $C_{15}H_{29}N$: 223.2293; observed, 223.2305.

RESULTS

Examination of the methylene chloride extracts of four collections (A–D) of *Diplorhoptrum* species from Puerto Rico revealed the presence of 2,6-dialkylpiperidines and 3,5-dialkylindolizidines **1–5** (Table 1, Figure 1), whose structures and stereochemistry were assigned by comparison with authentic samples and/or by spectroscopic methods.

Collection A. An extract from workers collected at El Verde, PR, contained *cis* and *trans*-2-methyl-6-nonylpiperidine (**1**) in ca. a 1 : 8 ratio, characterized by nearly identical mass spectra but differing gas chromatographic retention times, which can be compared to those of authentic samples prepared in earlier studies (MacConnell et al., 1974; Jones et al., 1982a,b; Blum et al., 1985). The occurrence of piperidines and the preponderance of the *trans* stereoisomer is typical of other *Solenopsis* species (Brand et al., 1972).

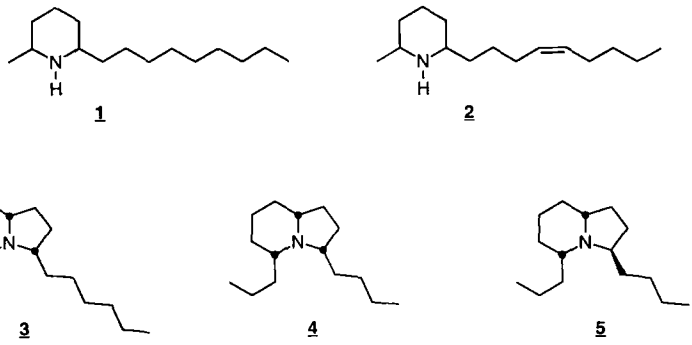
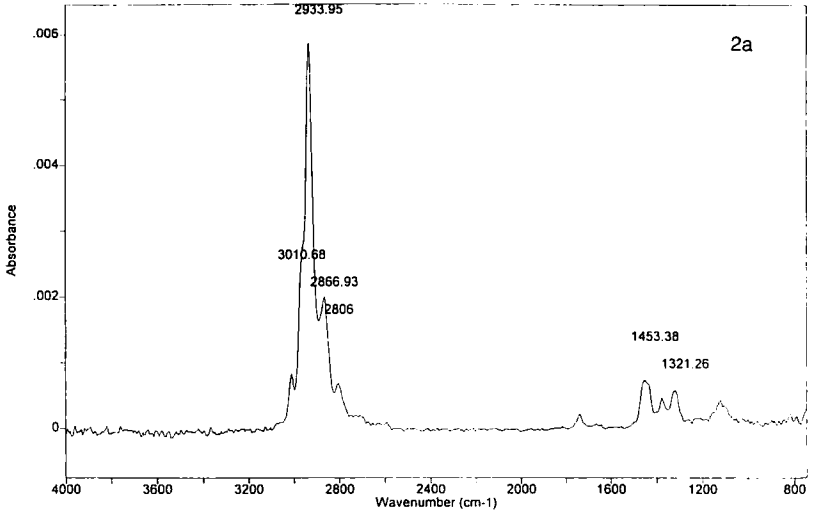


FIG. 1. Piperidines and indolizidines found in *Solenopsis* (*Diplorhoptrum*) species from Puerto Rico.

Collection B. An extract from workers collected at Río Grande, Puerto Rico, contained no alkaloids. The queens, however, contained the same alkaloids as above, *cis*- and *trans*-2-methyl-6-nonylpiperidine (**1**), but in ca. a 6:1 ratio. Additional confirmation that the major isomer was of the less commonly occurring ant alkaloid class of *cis* piperidines was provided by its FT-IR spectrum, which showed weak Bohlmann bands at 2803 and ca. 2730 cm^{-1} and was identical to that of a synthetic sample. These Bohlmann bands are absent in *trans* piperidines (Garraffo et al., 1994).

Collection C. An extract from workers collected at Mona Island, Puerto Rico, contained two components, **2a** and **2b,** in ca. a 1:4 ratio with virtually identical mass spectra: MS (*m/z*) (relative intensity), **2a** (minor): 223(M^+, 1), 222(1), 208(3), 180(5), 124(33), 111(15), 110(4), 99(7), 98(100), 96(8), 82(6), 81(8), 79(4), 70(9), 68(5), 69(27), 67(13), 56(10), 55(20); **2b** (major): 223(M^+, 2), 222(3), 208(5), 180(5), 124(15), 111(8), 99(8), 98(100), 81(4), 70(6), 69(4), 67(5), 57(8), 56(8), 55(13). The mass spectra indicated two unsaturated analogs of **1** that could be converted to a mixture of *cis*- and *trans*-**1** by catalytic hydrogenation (see Methods and Materials).

FT-IR spectra of **2a** and **2b** (Figure 2) indicated the first eluting component to be a *cis*-2,6-disubstituted piperidine and the second, its *trans* diastereomer. In addition, GC-FT-IR spectra showed a $\nu_{=CH}$ absorption at 3011 cm^{-1} and the absence of a terminal double bond as indicated by the lack of absorption at ca. 3080 ($\nu_{=CH}$ for a terminal olefin), 990 and 910 cm^{-1} ($\delta_{=CH}$ for a terminal olefin). An internal double bond of E stereochemistry was excluded by the absence of an absorption at ca. 964 cm^{-1} ($\delta_{=CH}$ for a *trans* olefin) and the presence of the 3011 cm^{-1} absorption, leaving only the possibility of an internal double bond of *Z* stereochemistry (cf. Attygalle, 1994). From the absence of



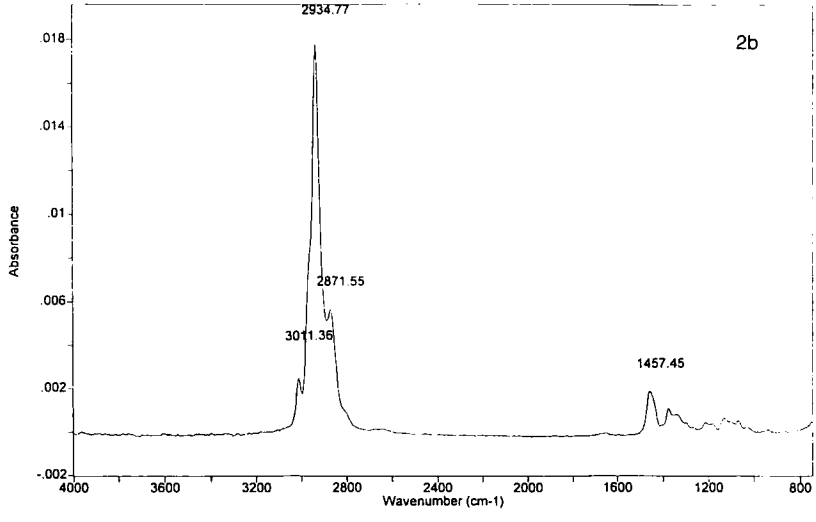


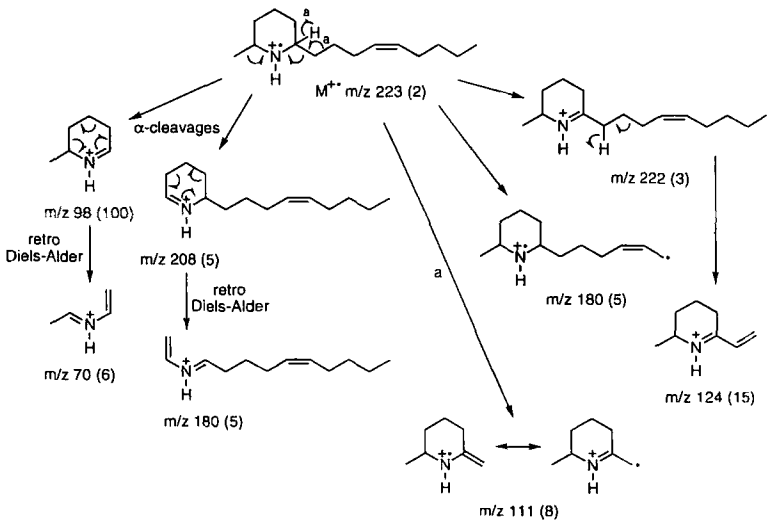
FIG. 2. GC-FT-IR spectra of the *cis*-ring piperidine **2a** and the *trans*-ring diastereomer **2b** from workers of *Diphlorhoptrum* sp. from Mona Island, Puerto Rico.

absorptions between 1600 and 1700 cm^{-1} in the infrared spectrum, the double bond in **2a/2b** was not imine or enamine unsaturation. Furthermore, vapor-phase FT-IR studies (Attygalle et al., 1994) on the position of the $\nu_{=CH}$ absorption on long-chain *Z*-monounsaturated primary acetates, excluded both Δ^{2-3} and Δ^{7-8} double bonds, since both show shifts to higher frequencies (ca. 3029 and 3022 cm^{-1}, respectively). A Δ^{3-4} position was also unlikely as their model indicated a 3018 cm^{-1} absorption for this structural class. Although the side-chain double bond in many ant venom alkaloids is in the terminal position, the loss of C_7H_{15} from the molecular ion on mass spectrometry, yielding an ion at *m/z* 124 (20%), suggested the double bond in **2a/2b** was nearer the piperidine ring and supported the structural inferences from the FT-IR spectra. Incidentally, the mass spectrum also ruled out a Δ^{1-2} position, as an *m/z* 96 base peak (with only a weak 98 peak), has been reported for pinidine [2-methyl-6-(1-propenyl)piperidine] (NIST Data Base, 1992).

We considered a Δ^{4-5} position as the most probable location of the *Z* double bond since a weak loss of C_3H_7 was seen from the molecular ion (*m/z* 223) to give a peak (5%) at *m/z* 180. Such a cleavage most probably arises from an allylic fragmentation. An analogous cleavage (M^+-heptyl, *m/z* 180) has been noted previously in dehydrosolenopsin-B, i.e., *trans*-2-methyl-6-(*Z*-4-tridecenyl)piperidine, a C_{19} homolog of **2b** from the venom of the red form of the fire ant, *Solenopsis invicta* (=*S. saevissma*) (MacConnell et al., 1971). Spectral data have been reported for the isomeric *cis*-2-methyl-6-(*Z*-4-tridecenyl)piperidine, where major fragments at *m/z* 124(20), 111(20) are also seen in addition to *m/z* 180(4) (Brand et al., 1972). In Scheme 1, we indicate probable fragmentation pathways to account for all major fragment ions observed in the mass spectra of **2a/2b.**

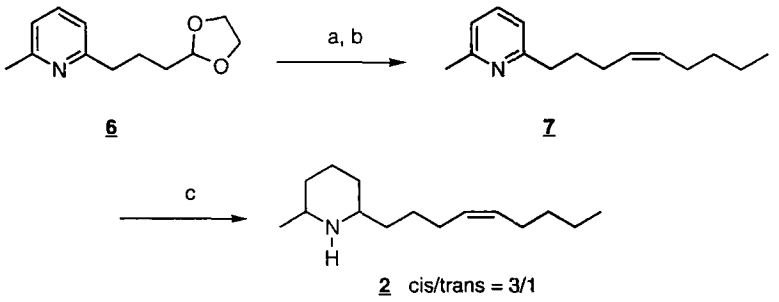
To prove this structure, the synthesis outlined in Scheme 2 was undertaken. The acetal intermediate **6** was synthesized from lutidine anion and 2-(2-bromoethyl)-1,3-dioxolane. This was deblocked to the aldehyde, which smoothly produced 2-methyl-6-(*Z*-4-nonenyl)pyridine (**7**) in a Wittig reaction (Johnson et al., 1982) with the phosphonium ylide (b) from 5-bromopentane. The olefin **7** was reduced, first with sodium in ethanol and then, after acidification to pH 6, by treatment with sodium cyanoborohydride to afford a mixture containing **2a** and **2b** (3.2:1, respectively). The GC retention times, and mass and GC-FT-IR spectra of the *cis* and *trans* disubstituted piperidines were identical in every respect to the natural alkaloids (see Methods and Materials). Coinjection on GC-MS of the synthetic mixture with the Mona Island workers' extract led to significant change in the ratio of **2a/2b** from the initial 1:4 to a 1:1.3 ratio, indicating each synthetic material to be identical in retention time to the corresponding natural material.

Along with traces of **2a/2b,** a 3-hexyl-5-methylindolizidine (**3**) was the predominant alkaloid in methylene chloride extracts from the queens of the



SCHEME 1. Proposed mass spectral fragmentations for **2b** (major isomer; *trans*-ring configuration); percentages in parentheses are electron-impact data obtained with an LKB-2091 instrument.

Mona Island collection. The mass spectrum [*m/z* 223 (M^+, 1), 222(1), 139(8), 138(100)] was the same as that reported for the compound found in the queens of an unidentified, but possibly identical, *Solenopsis species* collected in Marathon Key, Florida (Jones et al., 1984). The stereochemistry of **3** was established by examination of its FT-IR spectrum, which showed a broad range of Bohlmann bands decreasing in intensity from 2800 to 2500 cm^{-1}, which is characteristic



a) HCl/$HClO_4$, b) $Ph_3P^+C_5H_{11}$; BuLi, c) Na-EtOH; $NaCNBH_3$, pH 6

SCHEME 2. Chemical synthesis of piperidines **2a** and **2b**.

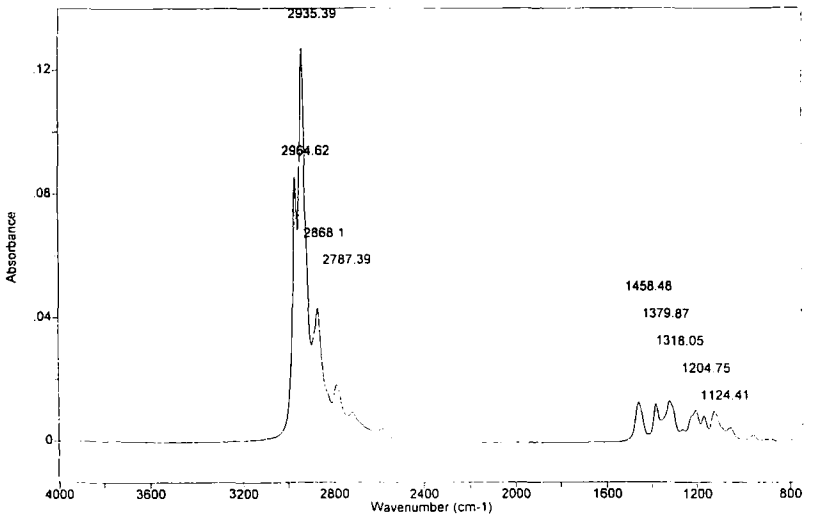


FIG. 3. GC-FT-IR spectrum of (5*Z*,9*Z*)-3-*n*-hexyl-5-methylindolizidine.

only of the 5*Z*,9*Z* isomer (Garraffo et al., 1993a). The *E*,*Z* nomenclature in the context of 3,5-disubstituted indolizidines refers to the hydrogen configurations at C-5 and C-9 with respect to that at C-3 and is either on the same molecular face (*Z*) or on the opposite face (*E*) (Sonnet et al., 1979). Compound **3** was identical with a synthetic sample of (5*Z*,9*Z*)-3-hexyl-5-methylindolizidine (Jones et al., 1984) (Figure 3). Indolizidines have been previously reported in various *Solenopsis* species (Jones et al., 1984). The Mona Island species is, at present, undescribed (Torres and Snelling, unpublished data).

Collection D. An extract from workers collected at Cabo Rojo, Puerto Rico, contained two components, **4** and **5**, in ca. 1:6 ratio, respectively, with the following mass spectra: (minor, **4**) MS *m/z*, 223(M^+, 2), 222(1), 180(30), 166(100), 124(5), 122(5); (major, **5**), MS *m/z*, 223(M^+, 4), 222(1), 180(100), 166(90), 124(7), 122(7). Extracts of two other populations of workers in the same area had ratios of these components of ca. 1:1.7 and 1:2.5. The mass and FT-IR spectra of **4** and **5** were identical to those reported for the amphibian alkaloids (5*Z*,9*Z*)-3-butyl-5-propylindolizidine and (5*E*,9*E*)-3-butyl-5-propylindolizidine, respectively (Garraffo et al., 1993a; Daly et al., 1993). Extracts from queens of the same colony contained trace amounts of **4** and **5** as well as one major component, which had mass and FT-IR spectra identical to those of **3**, the same indolizidine as found in queens from the Mona Island collection.

DISCUSSION

The alkaloids identified in this collection of *Diplorhoptrum* species from Puerto Rico may reflect the biological relationships in this group of ants. The unbranched 15-carbon skeleton of all these compounds is reminiscent of the unbranched 19-carbon alkaloid mixtures found in many New World and New Zealand *Monomorium* species (Jones et al., 1982b, 1988), where other carbon-chain lengths are not observed or are minor components. Additionally, while the presence of monocyclic alkaloids accompanying bicyclic alkaloids of the same carbon number in a particular ant venom has been reported, this is the first report of a monocyclic ant venom alkaloid (**2**) having side-chain functionality at a position that might permit the biosynthesis of a third C–N bond (in this case, by allylic activation), to yield the bicyclic alkaloid, **3**, found only in the queens. The *cis* ring diastereomer **2a**, detected as a minor component in Mona Island workers, would be the logical precursor.

Except for the queens of the Río Grande collection, where the unusual *cis*-**1** predominates, gas chromatographic retention times and FT-IR spectra show that the piperidine alkaloids **1** and **2** occur mainly as the usually encountered *trans* diastereomers with measurable amounts of *cis* diastereomers. It is noteworthy that the six-membered ring in the indolizidines **3, 4,** and **5** is *cis* disubstituted, of probable derivation from a *cis*-2,6-disubstituted piperidine, and suggesting biogenetic relationship with *Solenopsis conjurata*, whose venom contains *cis* and *trans* disubstituted piperidines along with a (5*Z*,9*Z*)-dialkylindolizidine (Jones et al., 1984).

In three of the four Puerto Rican *Diplorhoptrum* spp. for which queen and worker alkaloids have been compared (Table 1), significant differences are readily evident. Workers of the Río Grande species are extreme in not producing any detectable alkaloids in comparison with their queens. In addition, major quantitative and qualitative differences characterize the alkaloids synthesized by the Mona Island and Cabo Rojo species. Caste-specific differences in the mixtures of 2,6-dialkylpiperidines that dominate the venoms of fire ant (*Solenopsis geminata* group, sensu Trager spp.) have also been reported. For the most part, queens of these species produce a single diastereomeric pair of piperidines (*cis*- and *trans*-2-methyl-6-undecylpiperidine), whereas the workers of some species produce these and up to five pairs of *cis*/*trans* pyrrolidines (Brand et al., 1973). Since the queens are not actively involved in colony defense, their venoms may play another role. In the case of the queens of the Puerto Rican *Diplorhoptrum* spp., the presence of venom alkaloids not found in the workers strongly suggests a different function of these compounds in this caste, for example, as a pheromone or to protect the eggs from microbial infection (Vander Meer and Morel, 1994). A more systematic investigation of Puerto Rican *Diplorhoptrum* spp. is

being undertaken to determine the extent of chemical diversity in the venoms of these ants.

Most species of *Diplorhoptrum* are hypogeic, but some are known to be arboreal. An arboreal way of life may, in fact, be more common than generally realized, at least in tropical areas. Some of the species are facultative "thief ants," living in close proximity to nests of other, larger species. Often, their galleries may open into those of the larger ants. From these galleries, the *Diplorhoptrum* workers may raid the nest chambers of the other species for bits of food, but they are not above attacking the larvae and pupae of the unwilling host.

It is noteworthy that two of the four Puerto Rican *Diplorhoptrum* species studied here, the collections from Mona Island and Cabo Rojo, nest in trees, while the El Verde and Río Grande collections share the usual ground-nesting propensities of the group. An arboreal life may expose the workers to predators, such as lizards and frogs, which can readily forage on the tree and ingest large numbers of workers.

Rivero et al. (1963) found for the frog *Eleutherodactylus karlschmidti*, the most aquatic member of this genus in Puerto Rico, that most of its prey was terrestrial and 90% composed of insects. Forty-four percent of these, in turn, were comprised of dipterans, of which many were aquatic larvae. Trichopterans and hymenopterans each comprised about 15% of the insects consumed, and of the latter, most were ants. Lavigne and Drewry, who studied (1970) the prey of frogs and lizards at El Verde, found hymenopterans and dipterans to be dominant. Cintrón found (1970) a high consumption of coleopterans and hymenopterans (chiefly ants) by tree frogs of the genus *Eleutheradactylus* in palm (*Prestoea montana*) forests.

The present results are highly relevant to the proposal (Daly et al., 1994a,b) that dendrobatid frogs are sequestering, for defensive purposes, some of the venom components of dietary ants and other small arthropods. Thus, two further alkaloids (**4** and **5**), previously described only from dendrobatid frogs, have in the present paper been identified in an ant.

The indolizidine **5**, originally detected in dendrobatid frogs of the genus *Dendrobates* and named gephyrotoxin (GTX) **223AB**, has been the target of many syntheses (reviewed in Daly and Spande, 1986). It was originally thought that only the 5*E*,9*E* diastereomer occurred in dendrobatid frog skin based on the isolation of **5** from the Colombian species, *Dendrobates histrionicus* (Spande et al., 1981; Daly and Spande, 1986) while in the Panamanian species *Dendrobates speciosus* (Edwards et al., 1988) and one mantellid species from Madagascar (Garraffo et al., 1993b), only the 5*Z*,9*Z* diastereomer **4** was found. Additional work demonstrated that both **4** and **5** can also appear together in frog and toad skin extracts (Garraffo et al., 1993a; J. W. Daly, personal communication).

Dendrobatid frogs, raised in captivity on diets of fruit flies and crickets, have absolutely no skin alkaloids (Daly et al., 1992; 1994a,b) leading to the proposal that skin alkaloids are sequestered from dietary arthropods. Indeed, when fed fruit flies dusted with alkaloid-containing powder, such alkaloid-free frogs efficiently incorporated indolizidines, pyrrolizidines, and a number of other alkaloid classes (Daly et al., 1994a).

In addition, a captive-raised dendrobatid frog (*Dendrobates auratus*), when fed ants of the species *Monomorium pharaonis* (Daly et al., 1994a), incorporated the indolizidine, monomorine I, into skin. The frogs avidly ate these ants, so at least in this case the ant venom is not a deterrent to predation. These results indicate that the indolizidines **4** and **5** of the present paper should also be efficiently incorporated into dendrobatid frog skin.

At least half a dozen alkaloids of the indolizidine and pyrrolizidine classes have been found to be common to frogs and ants (Daly et al., 1993), strongly suggesting in the light of the above results that such alkaloids, when found in frog skin glands, have been sequestered from a diet containing ants. While this is not a large overlap—the total number of alkaloids detected in frog skins is over 400—it should be emphasized that only a few hundred of the many thousand species of myrmicine ants have so far been examined for alkaloids.

In Panama when captive-raised dendrobatid frogs were fed on leaf litter insects obtained through the use of Berlese funnels, some 20 alkaloids were found to have been sequestered into skin (Daly et al., 1994b). In this instance, no "ant" alkaloids were sequestered, perhaps suggesting that ants were poorly represented in the insects obtained from Berlese funnels. While the phenomenon of storing ingested alkaloids is not uncommon, having been encountered in a variety of vertebrate and invertebrate species, it may prove to be unusual in frogs, since only seven genera from four different families (Dendrobatidae, Bufonidae, Myobatrachidae, and Mantellidae) have lipophilic alkaloids with more than 80 genera examined to date. It is quite probable that this behavior reflects the presence of a transport and/or storage capacity for dietary alkaloids derived from ants or other small insects, but this has been demonstrated experimentally only for a few species of dendrobatid frogs, namely *Dendrobates auratus*, *Phyllobates bicolor*, and *Epipedobates tricolor* (Daly et al., 1994a,b).

Dendrobatid frogs are well known to include ants as a major food source (Donnelly, 1991; Toft, 1980); some dendrobatid frogs have even been referred to as "ant specialists" (Toft, 1995). *Solenopsis* species have been established by examination of stomach contents as among the food prey for frogs that are foreign to Puerto Rico (Silverstone, 1975; 1976). Ant species of the genus *Solenopsis* (*Diplorhoptrum*) related to the Puerto Rican species are sympatric with dendrobatids in Costa Rica (Jones et al., 1984) and are likely to be in other Central and South American areas as well. While the ant alkaloids have some taxonomic value, in that we have never observed collections of the same species

of ant with different alkaloids, in many cases, ants of different species do produce some of the same alkaloids, so that indolizidines **4** and **5** could very well be components of venoms of species not yet collected.

While certain ants serve as food for dendrobatid frogs, there are many that are rejected and shunned because of their bite or sting. It remains to be demonstrated, whether other classes of frog-skin alkaloids besides the 3,5-disubstituted indolizidines, **4** and **5**, will be found in ants that constitute the food of dendrobatid frogs. Dendrobatid frog skin also can contain precoccinelline, a beetle alkaloid, and a pyrrolizidine oxime closely related in structure to a millipede alkaloid (Daly et al., 1993). Indeed, such alkaloids were found in skin of dendrobatid frogs raised on leaf-litter insects in Panama (Daly et al., 1994b). Small beetles and millipedes have been documented as prey items for dendrobatid frogs and were present in the leaf litter collections.

It should be noted that almost all species of dendrobatid frogs are terrestrial. Only one of two arboreal Panamanian species contained even trace amounts of a putative ant alkaloid, in this case, alkaloid **195B**, 3-butyl-5-methylindolizidine (Daly et al., 1987). The correlation, if any, between terrestrial and arboreal ways of life of both insects and frogs in determining the skin alkaloids of the latter should be more thoroughly explored.

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CORN VOLATILES AS ATTRACTANTS FOR NORTHERN AND WESTERN CORN ROOTWORM BEETLES (COLEOPTERA: CHRYSOMELIDAE: *Diabrotica* SPP.)[1]

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Abstract—Synthetic corn volatiles and selected analogs were tested in commercial corn fields for attractiveness to feral northern (NCR, *Diabrotica barberi*) and western corn rootworm beetles (WCR, *D. virgifera virgifera*). Two new attractants, geranylacetone and α-terpineol, were identified among corn terpenes and compared at four stages in crop development with the phenylpropanoid standards cinnamyl alcohol and 4-methoxy-cinnamaldehyde, with each component at 30 mg/trap. Dose–response relationships (0.1–100 mg/trap) and efficacy of two-component blends (30 mg/component) were also examined. More beetles were captured on traps baited with (+)- than (−)-α-terpineol, but the difference was statistically significant only for WCR. Captures with geranylacetone or (+)-α-terpineol were directly proportional to the logarithm of the attractant dose. WCR females were attracted to as little as 0.1 mg of either compound. WCR males required ≥1.0 mg of (+)-α-terpineol and were not attracted to geranylacetone at any dose. NCR required ≥0.3 mg of either attractant and showed less marked response differences between the sexes than did WCR. Geranylacetone and cinnamyl alcohol were equally effective attractants, whereas (+)-α-terpineol was significantly less attractive to WCR but more attractive to NCR than was 4-methoxycinnamaldehyde. Corn terpenes and phenylpropanoid standards produced similar seasonal response patterns in that captures tended to rise in each case as the season progressed, except during silking when no compound was attractive. Mixing corn terpenes or phenylpropanoid standards synergized responses of WCR females, but (+)-α-terpineol suppressed attraction of NCR females to geranylacetone.

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Key Words—Coleoptera, Chrysomelidae, *Diabrotica barberi*, *Diabrotica virgifera virgifera*, *Zea mays*, corn rootworm, host finding, monoterpene, isoprenoid, attractant, kairomone, synergism, geranylacetone, α-terpineol.

INTRODUCTION

The northern corn rootworm (NCR), *Diabrotica barberi* Smith and Lawrence, and the western corn rootworm (WCR), *D. virgifera virgifera* LeConte, are the important diabroticite pests of corn, *Zea mays* L., in the United States, where pesticide costs and crop losses from corn rootworms have been estimated to be as high as $1 billion annually (Metcalf, 1986). Several types of semiochemicals that affect adult behaviors show promise for use in corn rootworm management programs (Levine and Oloumi-Sadeghi, 1991; Sutter and Lance, 1991; Metcalf and Metcalf, 1992). Among these semiochemicals are plant kairomones or parakairomones that apparently mimic host plant odors and play a role in feeding behavior (Andersen and Metcalf, 1986; Andersen, 1987; McGovern and Ladd, 1990; Metcalf and Lampman, 1991). Most of the known kairomonal attractants for corn rootworm adults are phenylpropanoids or benzenoids. These compounds have been identified mainly from plants of the family Cucurbitaceae, which serve as hosts for many *Diabrotica* spp. Although WCR and NCR beetles aggregate on cucurbit blossoms to feed on pollen and nectar, probably reflecting their ancestral relationship with this group of plants, both species are more closely associated with grasses, corn in particular, than with cucurbits (Metcalf et al., 1980; Branson and Krysan, 1981). The association is strongest for the larval stages, which are oligophagous on the Poaceae (previously Gramineae) and feed most successfully on corn.

Unidentified corn volatiles are known to attract both WCR and NCR adults (Prystupa et al., 1988) and may function in long-range orientation of these species to corn plants for both feeding and oviposition (Prystupa et al., 1988; Seigfried and Mullin, 1990). Several compounds attractive to corn rootworm beetles and present in squash blossoms, namely indole, β-ionone, 2-phenyl-1-ethanol, and phenylacetaldehyde (Andersen and Metcalf, 1986; Andersen, 1987; Lampman and Metcalf, 1987, 1988; Lampman et al., 1987; Metcalf and Lampman, 1989), also occur in corn (Thompson et al., 1974; Flath et al., 1978; Buttery et al., 1978, 1980; Cantelo and Jacobson, 1979). Considerable additional information exists on volatiles of corn plants (Thompson et al., 1974; Flath et al., 1978; Buttery et al., 1978, 1980; Cantelo and Jacobson, 1979; Buttery and Ling, 1984; Turlings et al., 1993; Takabayashi et al., 1995), but few of these compounds have been tested for attractiveness to corn rootworm beetles.

The present study tested volatile compounds known to occur in corn, as well as selected analogs, for attractiveness to feral NCR and WCR beetles in

commercial corn fields. The study further compared two newly discovered corn terpenoid attractants [geranylacetone, an irregular terpene, and (+)-α-terpineol, a monoterpene] with existing phenylpropanoid standards [cinnamyl alcohol and 4-methoxycinnamaldehyde (Metcalf and Lampman, 1989)] at four stages in crop development, established dose–response relationships for the terpenes, and examined two-component mixtures of either the terpene or standard attractants for synergistic and inhibitory effects on beetles captures.

METHODS AND MATERIALS

Figure 1 gives the structure and purity of test compounds. α-Terpineol, (*E*)-6,10-dimethyl-5,9-undecadien-2-one (=geranylacetone), (*E*,*E*)-2,4-decadienal, limonene, and citral were obtained from Fluka Chemical Corp., Ronkonkoma, New York. 4-Methoxycinnamaldehyde was obtained from Schweizerhall Inc., South Plainfield, New Jersey, and all others were from Aldrich Chemical Co., Milwaukee, Wisconsin. The compounds were applied for testing to cotton dental rolls (3.8 cm long × 1 cm diam.) (Patterson Dental Co., Minneapolis, Minnesota) that were treated with 0.4 ml of a 1:1 mixture

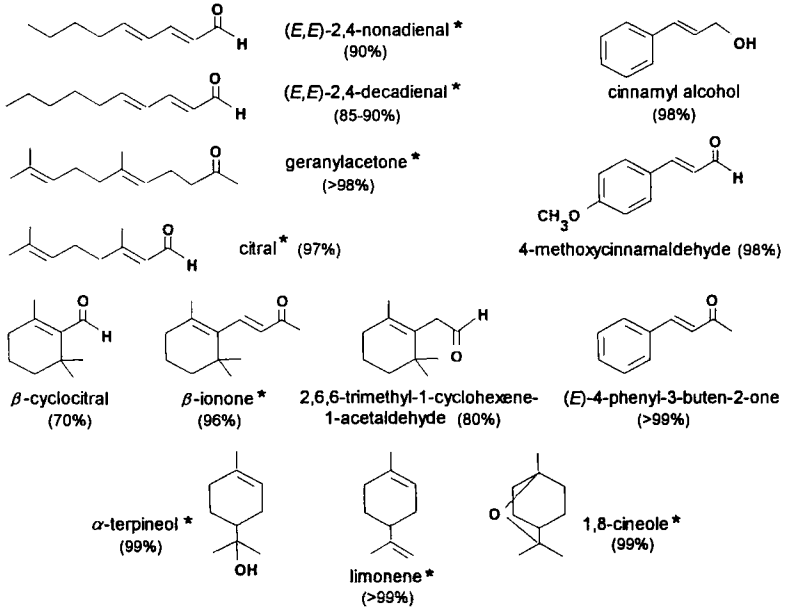

FIG. 1. Structure and purity (in parentheses) of test compounds. Asterisks indicate compounds identified from corn (see text for references).

of olive oil in hexane to sustain volatilization (Guss, 1976). The rolls were then affixed to yellow Pherocon A.M. sticky traps (Trécé Inc., Salinas, California), which were placed in 1993 or 1994 in commercial corn fields that were located in Brookings County, South Dakota, and had been in continuous corn for at least two years. Control traps were baited with dental rolls that were treated only with the oil–hexane mixture and acetone, which was used to solubilize and dilute candidate attractants. The acetone volume per trap varied among tests but was held constant among treatments within tests.

Individual candidate attractants were initially tested at a dose of 100 mg/trap, including stereoisomers of α-terpineol, which were also subsequently compared at a dose of 30 mg/trap in a test conducted August 29–31, 1994. Seasonal variation in response to the new terpenoid attractants was compared with that to the phenylpropanoid standards at a dose of 30 mg/trap by repeating tests during four stages in crop phenology (Ritchie et al., 1992): V10+ (late vegetative), R1 (silking), R3–R4 (late milk to early dough), and R4–R5 (late dough to early dent). Dose–response relationships were established for the terpenes in a test that included a series of (+)-α-terpineol and geranylacetone treatments, each ranging from 0.1 to 100 mg/trap, and a control. The effect on beetle captures of mixing attractant chemicals [geranylacetone mixed with (+)-α-terpineol and cinnamyl alcohol with 4-methoxycinnamaldehyde] was determined by dispensing 30 mg of each chemical from the same and from separate traps. When traps received both chemicals, each chemical was placed on a separate half of a dental wick that had first been treated with 0.2 ml of the 1 : 1 mixture of olive oil in hexane. Each half of the wick was then affixed separately to the trap to minimize the likelihood of chemical reactions between test compounds that were volatilized from the same trap.

Each experiment was laid out in a randomized complete block design, with six replicates in the initial screening of candidate attractants at a dose of 100 mg/trap and in the dose–response test, or eight replicates in all other tests, including comparisons repeated seasonally. Traps were wrapped around corn plants at about ear height and were separated within blocks by 30 m. Blocks were laid out within a corn row and were at least 30 m from one another and from the edge of the field. The number of NCR and WCR beetles visible per corn plant and corn developmental stage were determined during each experiment in a sample of 40 plants situated throughout the entire test area at positions in trap rows about equidistant between traps. Traps were left in the field for 48 hr, with two exceptions: cold rainy weather during the first several days of the dose–response test resulted in a six-day test period and the effect of volatilizing 4-methoxycinnamaldehyde and cinnamyl alcohol from the same trap was evaluated after a 24-hr trapping period. Traps were then returned to the laboratory, where counts were made by sex of the numbers of NCR and WCR beetles caught.

Insect captures on traps baited with single components of a two-component mixture were summed across treatments and this total compared with the sum of captures on doubly-baited and control traps to determine whether mixing attractants synergized or inhibited insect responses, i.e., produced responses significantly higher or lower than the sum of responses to individual compounds. This comparison is expressed as a ratio of interaction (ROI) = [(A + B) + Control]/[(A) + (B)], where (A) is the capture on a trap baited with compound A, (B) the capture on a trap baited with compound B, (A + B) the capture on a trap baited with both compounds, and control the capture on an unbaited trap. Captures on doubly-baited traps were increased by control captures to account for the twofold difference between one- and two-component treatments in the number of traps deployed and to avoid a possible negative or zero denominator had adjustments been made by subtraction of control captures from those on baited traps. An ROI significantly above or below or equal to 1 provided evidence for synergistic, inhibitory, or additive interactions, respectively, between test compounds.

To determine whether ROI values differed significantly from 1, a two-tailed t test for paired samples was used to test the null hypothesis of no difference between ROI numerator and denominator. Except for these t tests and the linear regression analyses described below for the dose–response test, data from each experiment were transformed as $\ln(x + 1)$ to normalize variances and were then analyzed by ANOVA. The Student-Newman-Keuls test was used to separate multiple means after a significant ANOVA. $P \leq 0.05$ was considered statistically significant. Untransformed means and standard errors are presented in the tables. For each attractant in the dose–response test, mean responses were calculated for each test dose, and the relationship between the logarithm of the attractant dose and the mean response was analyzed by linear regression. Mean responses not significantly different from control were omitted from each regression analysis, except for inclusion of that mean associated with the highest dose that failed to produce a significant response. Statistical analyses were performed with ANOVA, MEANS, and REG procedures of the SAS Institute (1988a,b).

RESULTS

The initial screening of individual candidate attractants clearly indicated an increased capture of corn rootworm adults compared with controls when traps were baited with 100 mg of geranylacetone or (+)-α-terpineol (Tables 1 and 2, respectively). Both corn rootworm species reacted to both compounds, although NCR appeared more responsive to geranylacetone than to (+)-α-terpineol, whereas WCR showed the opposite pattern. Both NCR sexes were attracted, whereas only female WCR responded above control levels. (+)-α-Terpineol

TABLE 1. MEAN CAPTURE ± SE OF NORTHERN AND WESTERN CORN ROOTWORM ADULTS ON TRAPS BAITED WITH CANDIDATE ATTRACTANTS[a]

Candidate attractant (100 mg/trap)	Northern corn rootworm		Western corn rootworm	
	Females	Males	Females	Males
(*E*,*E*)-2,4-Nonadienal	1.2 ± 0.4 b	15.3 ± 3.8 b	1.0 ± 0.5 d	9.0 ± 4.6
(*E*,*E*)-2,4-Decadienal	0.8 ± 0.4 b	18.0 ± 3.7 b	0.5 ± 0.2 d	7.7 ± 1.7
Geranylacetone	69.2 ± 6.7 a	104.3 ± 14.0 a	12.5 ± 2.2 b	7.7 ± 2.5
Citral	1.3 ± 0.2 b	17.7 ± 4.2 b	1.7 ± 1.1 d	3.5 ± 1.4
β-Cyclocitral	2.3 ± 1.2 b	13.0 ± 2.1 b	4.5 ± 0.6 c	5.0 ± 1.4
β-Ionone	2.2 ± 0.5 b	19.8 ± 3.0 b	37.0 ± 11.7 a	10.0 ± 2.4
2,6,6-Trimethyl-1-cyclohexene-1-acetaldehyde	0.5 ± 0.3 b	16.0 ± 4.1 b	1.7 ± 0.5 cd	4.2 ± 1.2
(*E*)-4-Phenyl-3-buten-2-one (benzalacetone)	1.8 ± 0.9 b	22.5 ± 5.6 b	2.8 ± 1.1 cd	8.8 ± 2.0
Control (none)	1.5 ± 0.8 b	22.5 ± 3.3 b	0.5 ± 0.2 d	7.0 ± 1.9
F statistic[b]	25.88**	10.84**	20.40**	1.29 NS

[a]Test conducted September 8–10, 1993. Mean number ± SE of northern and western corn rootworm beetles counted per corn plant on September 9 was 1.6 ± 0.2 and 0.6 ± 0.1, respectively. Corn was in the milk to dough stage (R3–R4).

[b]Asterisks denote statistical significance at $P < 0.0001$, NS denotes $P > 0.05$ ($df = 8, 40$). Means within a column followed by the same letter do not differ by Student-Newman-Keuls test, $P > 0.05$.

TABLE 2. MEAN CAPTURE ± SE OF NORTHERN AND WESTERN CORN ROOTWORM ADULTS ON TRAPS BAITED WITH CANDIDATE ATTRACTANTS[a]

Candidate attractant (100 mg/trap)	Northern corn rootworm		Western corn rootworm	
	Females	Males	Females	Males
(+)-α-Terpineol	20.8 ± 3.6 a	32.0 ± 4.7 a	116.7 ± 26.0 a	9.2 ± 1.5
(−)-α-Terpineol	14.5 ± 1.3 a	27.2 ± 6.0 a	61.3 ± 14.0 b	8.2 ± 1.2
(*R*)-(+)-Limonene	2.8 ± 1.4 b	11.8 ± 2.7 b	4.3 ± 0.7 c	6.8 ± 1.8
(*S*)-(−)-Limonene	1.7 ± 0.5 b	9.0 ± 2.2 b	3.3 ± 1.4 c	4.7 ± 1.2
1,8-Cineole	2.3 ± 0.6 b	13.2 ± 2.5 b	4.8 ± 1.2 c	4.7 ± 1.6
Control (none)	2.8 ± 0.7 b	13.5 ± 2.2 b	3.0 ± 0.9 c	5.8 ± 1.7
F statistic[b]	19.16**	9.34**	61.01**	1.70 NS

[a]Test conducted September 15–17, 1993. Mean number ± SE of northern and western corn rootworm adults counted per corn plant on September 15 was 0.4 ± 0.1 and 0.7 ± 0.2, respectively. Corn was in the dent stage (R5).

[b]Asterisks denote statistical significance at $P < 0.0001$, NS denotes $P > 0.05$ ($df = 5, 25$). Means within a column followed by the same letter do not differ by Student-Newman-Keuls test, $P > 0.05$.

attracted more of both species than did (−)-α-terpineol, although only the WCR difference in response to the stereoisomers was statistically significant (Table 2). A similar pattern occurred when the dose of (+)- and (−)-terpineol was reduced to 30 mg/trap (respectively, mean trap captures ± SE for WCR of 67.5 ± 12.8 vs. 38.6 ± 3.0 and for NCR of 27.8 ± 7.3 vs. 22.1 ± 4.3).

WCR females were also significantly, albeit weakly, attracted by β-cyclocitral (Table 1); however, purity of the test sample was low (70%), and the slight response might have been caused by unknown contaminants. As expected from an earlier study (Lampman and Metcalf, 1988), β-ionone attracted significantly more WCR females than did controls. None of the following compounds was attractive: (*E*,*E*)-2,4-nonadienal, (*E*,*E*)-2,4-decadienal, citral, 2,6,6-trimethyl-1-cyclohexene-1-acetaldehyde, (*E*)-4-phenyl-3-buten-2-one, limonene, or 1,8-cineole (Tables 1 and 2).

When beetle captures on traps baited with geranylacetone, cinnamyl alcohol, (+)-α-terpineol, and 4-methoxycinnamaldehyde were compared at four stages in crop phenology (Tables 3 and 4 for NCR and WCR, respectively), no test compound was attractive during corn silking, but NCR and WCR captures otherwise tended to increase as the season progressed, regardless of test compound, and to do so sooner in females than in males. Counts of WCR and NCR on plants also increased as the season progressed (Tables 3 and 4), although more moderately than increased captures on attractant-baited traps. The low WCR plant counts during presilking probably accounted for the low WCR captures early season.

Geranylacetone proved as attractive as cinnamyl alcohol to NCR throughout the season (Table 3), whereas (+)-α-terpineol was clearly less effective than 4-methoxycinnamaldehyde for WCR (Table 4). Geranylacetone again attracted NCR of both sexes and was moderately attractive to WCR females, but was not attractive to males late in the season when corn was in the late dough to early dent stage. (+)-α-Terpineol trapped more WCR females than did geranylacetone, but attractancy again occurred only late in the season. NCR females showed a significant, albeit weak, response to (+)-α-terpineol but not to 4-methoxycinnamaldehyde late in the season. Neither (+)-α-terpineol nor 4-methoxycinnamaldehyde attracted NCR males in this test series.

Figure 2 presents mean captures per trap of WCR and NCR as a function of the logarithm of the dose of geranylacetone or (+)-α-terpineol used to bait the traps. Mixed-sex captures are reported, except that the WCR response to geranylacetone is based only on females because WCR males failed to discriminate baited from control traps at all geranylacetone test doses, and use of mixed-sex captures would have obscured the significant relationship between geranylacetone dose and the response of WCR females. Given this stipulation, captures of both species increased with dose for both attractants and were maximum at

TABLE 3. MEAN CAPTURE ± SE OF NORTHERN CORN ROOTWORM ADULTS ON ATTRACTANT-BAITED TRAPS DURING FOUR STAGES OF CORN DEVELOPMENT[a]

Sex	Attractant (30 mg/trap)	Late vegetative (V10+) July 15–17	Silking (R1) July 27–29	Milk to dough (R3-R4) Aug. 12–14	Dough to dent (R4-R5) Aug. 26–28
Female	Geranylacetone	18.6 ± 7.3 a	0.3 ± 0.2	55.3 ± 8.1 a	99.8 ± 19.2 a
	Cinnamyl alcohol	9.5 ± 2.8 a	0.4 ± 0.3	32.9 ± 5.3 a	108.0 ± 14.2 a
	(+)-α-Terpineol	0.3 ± 0.2 b	0.4 ± 0.2	3.0 ± 0.6 b	13.9 ± 3.2 b
	4-Methoxycinnamaldehyde	0 b	0.3 ± 0.3	5.0 ± 1.3 b	2.9 ± 0.4 c
	Control (none)	0.3 ± 0.2 b	0	4.8 ± 1.2 b	3.5 ± 0.9 c
	F statistic[b]	31.31**	0.50 NS	34.94**	74.07**
Male	Geranylacetone	19.5 ± 4.3 a	10.5 ± 3.3	29.3 ± 7.9	76.3 ± 11.2 a
	Cinnamyl alcohol	20.6 ± 2.8 a	10.5 ± 2.6	23.6 ± 4.1	54.9 ± 7.3 a
	(+)-α-Terpineol	2.8 ± 0.5 b	10.6 ± 3.6	23.5 ± 8.4	27.5 ± 2.3 b
	4-Methoxycinnamaldehyde	1.6 ± 0.6 b	6.8 ± 1.7	27.4 ± 5.5	22.0 ± 3.3 b
	Control (none)	1.6 ± 0.5 b	5.8 ± 1.3	22.3 ± 5.0	24.8 ± 7.6 b
	F statistic[b]	46.73**	0.62 NS	0.37 NS	14.22**

[a] Mean number ± SE of northern corn rootworms counted per corn plant during each trapping interval was 0.9 ± 0.3, 1.6 ± 0.4, 2.2 ± 0.4, and 2.8 ± 0.3, respectively, listed in order from early- to late-season 1994 trapping interval.

[b] Asterisks denote statistical significance at $P < 0.0001$, NS denotes $P > 0.05$ ($df = 4, 28$). Means within sexes and columns followed by the same letter do not differ by Student-Newman-Keuls test, $P > 0.05$.

TABLE 4. MEAN CAPTURE ± SE OF WESTERN CORN ROOTWORM ADULTS ON ATTRACTANT-BAITED TRAPS DURING FOUR STAGES OF CORN DEVELOPMENT[a]

Sex	Attractant (30 mg/trap)	Late vegetative (V10+) July 15–17	Silking (R1) July 27–29	Milk to dough (R3-R4) Aug. 12–14	Dough to dent (R4-R5) Aug. 26–28
Female	Geranylacetone	0	0	2.9 ± 1.1 b	50.0 ± 15.6 c
	Cinnamyl alcohol	0.1 ± 0.1	0	2.5 ± 0.5 b	21.6 ± 3.1 c
	(+)-α-Terpineol	0	0	4.0 ± 1.1 b	122.8 ± 15.5 b
	4-Methoxycinnamaldehyde	0.4 ± 0.3	0	63.4 ± 16.9 a	535.9 ± 31.7 a
	Control (none)	0.1 ± 0.1	0	2.4 ± 0.6 b	7.5 ± 2.3 d
	F statistic[b]	1.02 NS	not done	41.5**	48.76**
Male	Geranylacetone	0 b	1.1 ± 0.5	9.4 ± 1.8	28.4 ± 6.7 b
	Cinnamyl alcohol	0.1 ± 0.1 b	0.5 ± 0.2	12.9 ± 3.1	29.0 ± 7.0 b
	(+)-α-Terpineol	0.4 ± 0.3 b	1.0 ± 0.5	9.3 ± 1.3	24.0 ± 2.8 b
	4-Methoxycinnamaldehyde	1.5 ± 0.5 a	1.4 ± 0.6	18.6 ± 5.9	106.5 ± 10.6 a
	Control (none)	0.6 ± 0.3 ab	1.3 ± 0.4	13.0 ± 3.3	31.0 ± 7.1 b
	F statistic[b]	5.43*	0.53 NS	0.95 NS	14.35**

[a] Mean number ± SE of western corn rootworms counted per corn plant during each trapping interval was 0.03 ± 0.03, 0.4 ± 0.1, 0.9 ± 0.2, and 1.5 ± 0.2, respectively, listed in order from early- to late-season 1994 trapping interval.

[b] Asterisks denote statistical significance at $*P < 0.01$ or $**P < 0.0001$, NS denotes $P > 0.05$ ($df = 4, 28$). Means within sexes and columns followed by the same letter do not differ by Student-Newman-Keuls test, $P > 0.05$.

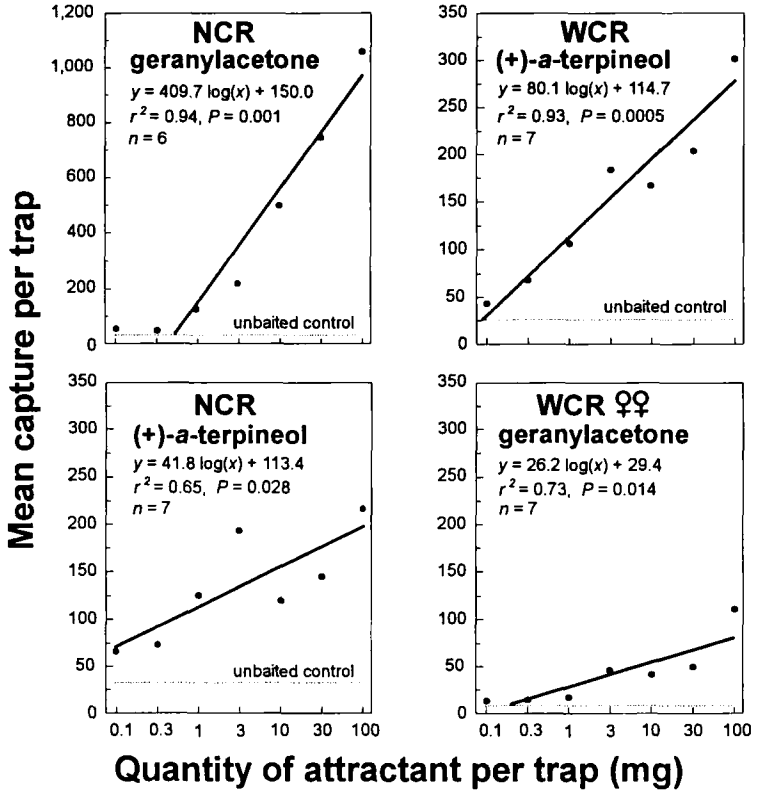

FIG. 2. Relationship between attractant dose plotted logarithmically and capture of northern (NCR) and western (WCR) corn rootworm beetles on attractant-baited traps. The six-day test ended September 6, 1994, when corn was dough to dent stage (R4–R5) and beetle counts per corn plant (mean ± SE) were 1.3 ± 0.2 for NCR and 0.6 ± 0.1 for WCR.

100 mg/trap. Attractant dose plotted logarithmically accounted for 93% or more of the variation in mean responses when NCR and WCR were tested with geranylacetone and (+)-α-terpineol, respectively. Lower R^2 values occurred when mixed-sex NCR reacted to (+)-α-terpineol (0.65) and WCR females reacted to geranylacetone (0.73). In the former case, nearly maximum captures at a dose of only 3.0 mg/trap raised the possibility of a plateau in NCR responses at the higher (+)-α-terpineol doses. The lowest dose to produce a mixed-sex response significantly higher than control was 0.3 mg/trap for NCR and WCR tested with (+)-α-terpineol, 1.0 mg/trap for NCR tested with geranylacetone,

and 3.0 mg/trap for WCR tested with geranylacetone. Detection thresholds did not vary with male, female, and mixed-sex NCR groupings, except that (+)-α-terpineol was first detected at a dose of 0.3 mg/trap by males and mixed-sex groups and at 1.0 mg/trap by females. WCR females responded at lower doses, and as little as 0.1 mg/trap of (+)-α-terpineol or geranylacetone produced captures significantly higher than those to controls. In contrast, WCR males were not attracted to (+)-α-terpineol at doses below 1.0 mg/trap and failed to respond even to 100 mg of geranylacetone. Sex also appeared to strongly influence slope (±SE) of the log dose–response lines in WCR but not NCR [for females compared with males: 61.7 ± 11.0 compared with 17.1 ± 5.0 for WCR responding to (+)-α-terpineol, 26.2 ± 7.1 and no significant male responses for WCR tested with geranylacetone, 17.7 ± 4.6 and 25.9 ± 11.1 for NCR responding to (+)-α-terpineol, and 183.2 ± 39.2 and 226.5 ± 22.6 for NCR responding to geranylacetone. These slopes differed significantly from 0, except for the 25.9 value for NCR males responding to (+)-α-terpineol ($P = 0.07$).]

When efficacy of two-component blends was compared with that of individual components of each blend through calculation of the ratio of interaction (ROI) (Table 5), only females produced ROI values that differed significantly from 1. WCR females produced ROI values that were significantly higher than 1, whether tested with geranylacetone and (+)-α-terpineol (1.71) or cinnamyl alcohol and 4-methoxycinnamaldehyde (1.57). NCR females produced a similarly elevated ROI value of 1.64 to the latter blend, but higher variability precluded statistical significance ($P = 0.27$). An ROI significantly less than one (0.81) for NCR females tested with the blend of (+)-α-terpineol and geranylacetone indicated inhibitory interaction, and (+)-α-terpineol at the 30 mg/trap dose appeared to suppress attraction to geranylacetone.

DISCUSSION

This study is the first to show geranylacetone and α-terpineol attractive to NCR and WCR beetles. These two terpenes are volatile constituents of several parts of corn plants consumed by corn rootworm adults. α-Terpineol occurs in silks (Flath et al., 1978) and geranylacetone is present in kernels and husks (Buttery et al., 1978). Adults of both corn rootworm species prefer a diet of corn pollen, fresh silks, and young kernels. These foods become scarce as corn matures and WCR increasingly utilize corn leaves, whereas NCR tend to migrate out of corn fields to feed on pollen from a variety of other plants before returning to corn for oviposition (Ludwig and Hill, 1975; Branson and Krysan, 1981; Boetel et al., 1992).

Because geranylacetone and α-terpineol were isolated from corn via vacuum steam distillation (Buttery et al., 1978; Flath et al., 1978) and this method

TABLE 5. OLFACTORY INTERACTIONS FOR NORTHERN (NCR) AND WESTERN CORN ROOTWORM (WCR) ADULTS BETWEEN CHEMICALS IN TWO-COMPONENT ATTRACTANT BLENDS DETERMINED FROM CAPTURES WHEN 30 mg OF EACH COMPONENT WERE DISPENSED FROM SAME VS. SEPARATE TRAPS[a]

Chemicals	Species and sex	Capture/trap (mean ± SE)				Ratio of interaction (ROI)[b]
		A	B	A + B	Control	
A = Geranylacetone	WCR female	13.1 ± 2.1	19.4 ± 3.9	36.1 ± 4.4	11.8 ± 3.7	1.71 ± 0.33*
B = (+)-α-Terpineol	WCR male	20.6 ± 4.5	20.1 ± 5.2	18.4 ± 2.8	16.0 ± 5.1	0.90 ± 0.10
	NCR female	42.1 ± 7.8	6.0 ± 0.9	31.0 ± 5.0	4.1 ± 1.4	0.81 ± 0.14*
	NCR male	37.3 ± 7.1	19.3 ± 5.0	27.1 ± 3.4	16.5 ± 3.2	0.85 ± 0.09
A = 4-Methoxy-	WCR female	181.4 ± 31.1	11.1 ± 3.8	280.3 ± 45.3	3.0 ± 0.6	1.57 ± 0.23*
cinnamaldehyde	WCR male	39.8 ± 3.7	13.6 ± 3.9	40.1 ± 3.2	10.5 ± 1.9	1.07 ± 0.17
B = Cinnamyl alcohol	NCR female	4.1 ± 1.5	42.1 ± 9.1	57.0 ± 9.1	2.0 ± 0.9	1.64 ± 0.35
	NCR male	10.4 ± 2.5	15.1 ± 2.6	17.0 ± 2.7	5.8 ± 1.2	1.05 ± 0.18

[a]Tests were started August 24, 1994. Mean number ± SE of NCR and WCR per corn plant was 3.1 ± 0.5 and 1.1 ± 0.2 on August 26 (terpene test) and 5.1 ± 0.6 and 1.2 ± 0.2 on August 24 (phenylpropanoid test), respectively. Corn was in the dough stage (R4) August 24 and 26.
[b]Mean = SE; *$P < 0.05$ that ROI = 1 by a paired two-tailed t test ($df = 7$).

may detect volatile compounds not actually released from plants, the occurrence of the two terpenes in the headspace around corn plants in late vegetative and reproductive stages remains to be demonstrated. Nevertheless, the presence of these terpenes in corn distillate and their attractiveness in my tests to feral corn rootworm beetles of both sexes, especially at the lower doses used in the dose–response test, suggest that they are kairomonal feeding attractants of corn plant origin. A role in ovipositional behavior is also possible given the close association between feeding and ovipositional behaviors in diabroticite beetles, especially in WCR (Branson and Krysan, 1981).

Neither terpene is unique to corn; for example, α-terpineol and probably geranylacetone occur in sunflowers (Etievant et al., 1984), one of a number of flowering forbes that NCR use for food late in the season once corn silks have dried (Cinereski and Chiang, 1968; Krysan and Branson, 1983). α-Terpineol also occurs in evergreens and is attractive to pine beetles and several scolytid bark beetles (see Brattsten, 1983). As a group, plant terpenoids are important intermediaries in diverse insect–plant interactions and may function among other roles as feeding or oviposition deterrents, stimulants, and attractants, including herbivore-induced synomones mediating tritrophic interactions (Brattsten, 1983; Coats et al., 1991; Chou and Mullin, 1993; Dicke, 1994). Despite instances of attraction, terpenes often afford plants an effective chemical defense against herbivory (Brattsten, 1983; Coats et al., 1991).

Several groups of compounds in addition to the corn terpenoid attractants evoke positive responses from *Diabrotica* spp., even though the same groups frequently function as toxic allelochemicals in other insect–plant interactions. In this category are cucurbitacins, triterpenoid defensive compounds typically of cucurbit origin that elicit arrest and compulsive feeding in diabroticites on contact but are highly unpalatable as well as toxic to a wide variety of other organisms (Metcalf et al., 1980). Chemicals generally defending against herbivory are also represented among the compounds that attract WCR larvae toward corn roots [i.e., 6-methoxy-2-benzoxazolinone or MBOA (Bjostad and Hibbard, 1992; Hibbard et al., 1995)].

Although α-terpineol and geranylacetone are common constituents of floral odors (Knudsen et al., 1993), neither chemical was reported among volatile constituents of squash blossoms, where kairomones attractive to corn rootworm adults have so far been sought (Andersen and Metcalf, 1986, 1987; Andersen, 1987). Unlike the corn terpenoid baits, attractants of squash-blossom origin (and their many active analogs) generally contain a phenyl ring. The sole exception is β-ionone, an irregular terpene that shares the cyclohexene structure of α-terpineol and is present in steam distillates of both corn and squash (Buttery et al., 1978, 1980; Flath et al., 1978; Andersen, 1987). Geranylacetone is unique in that it lacks the six-carbon ring invariably present in other reported kairomonal or parakairomonal attractants for adult corn rootworms. Some sim-

ilarity between geranylacetone and β-ionone can be visualized by breaking the cyclohexene ring of β-ionone between the 1 and 6 positions.

Despite the structural novelty of the corn terpenes among known kairomonal attractants of corn rootworm adults, the terpenes nevertheless produced a seasonal response pattern in NCR and WCR similar to that of the phenylpropanoid standards. Captures with both groups of compounds tended to increase as the season progressed, except during corn silking when no compound was effective. Previous studies have also reported diminished or insignificant captures of corn rootworm beetles with phenylalkanoid-baited traps around the time of corn flowering, as well as a tendency for higher trap catches late season (Andersen and Metcalf, 1986; Lampman et al., 1987; Lance and Elliott, 1991; Hesler et al., 1994; Hammack and Hesler, 1995). This pattern could result from seasonal variation in beetle responsiveness to attractants or from seasonal variation in the abundance of natural food sources, most probably combined with poor competition of single-component lures with natural scents that are almost certainly complex mixtures. Preferred foods such as corn pollen and succulent silks are most abundant around the time of flowering, when single-component lures were least effective.

Sex, species, and dose all interacted to affect capture of corn rootworm adults on traps baited with the terpenoid attractants. Even though NCR captures were about five times higher with geranylacetone than with (+)-α-terpineol at the higher doses, the NCR response threshold was actually slightly lower for (+)-α-terpineol than for geranylacetone. The dose–response data suggested that NCR captures peaked at about 3 mg of (+)-α-terpineol, and this is consistent with the finding that 30 mg of (+)-α-terpineol significantly suppressed responses of NCR females to 30 mg of geranylacetone. Although captures on control traps tended to be higher for males than females, perhaps reflecting greater random movement of males at trap height, sex had little effect in NCR on slopes of dose–response lines or response thresholds to the terpenes. That was not true for WCR, and WCR females were clearly the more responsive sex, or even the only sex to respond, as in the case of geranylacetone. WCR males showed a dose-dependent response to (+)-α-terpineol in the dose–response test, but no significant male response was recorded in other tests with this compound. This discrepancy might be explained by the longer test period (six days rather than two) in the former test, perhaps combined with a test date late in the season when captures were found to be highest.

Corn volatiles known to attract diabroticite beetles are widely distributed among plants; for example, α-terpineol, geranylacetone, β-ionone, indole, and phenylacetaldehyde all occur in *Acacia* blossoms (Flath et al., 1983) and, as already noted, the first two compounds are probably present in sunflowers (Etievant et al., 1984). Thus, it is unlikely that these compounds can account for specificity of the host-finding responses of corn rootworm adults, except as constituents of a distinctive odorant blend. Several mixtures formulated from

squash-blossom volatiles are known to elicit synergistic rather than additive attraction responses from diabroticite beetles; these mixtures include VIP (equal parts by weight of veratrol, indole, and phenylacetaldehyde) for southern corn rootworms (SCR), *D. undecimpunctata howardi* Barber, and TIC [equal parts by weight of 1,2,4-trimethoxybenzene, indole, and (*E*)-cinnamaldehyde] for NCR, WCR, and SCR (Lampman and Metcalf, 1987; Metcalf et al., 1995). The present study also indicated at least modest synergism in the response of WCR females to two-component blends of corn terpenes, as well as phenylpropanoid standards, and suggested that NCR may be sensitive to ratios of attractants comprising a blend. That is, a blend of the two corn terpenes may prove more effective for NCR when (+)-α-terpineol is a considerably more minor component than geranylacetone.

Further examination of interactions among volatiles attractive to corn rootworm adults is clearly warranted, using blends that are varied both qualitatively and quantitatively. Studies aimed at defining the blend of compounds released from corn plants at stages fostering corn rootworm adult feeding and oviposition would provide a valuable framework for examining such interactions and likely yield additional behaviorally active compounds. More detailed information about the identity of corn-produced kairomones and their precise roles in host-finding, feeding, and ovipositional behaviors of diabroticite adults is needed and may lead to powerful new tools for corn rootworm management (Levine and Oloumi-Sadeghi, 1991).

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CONTROL OF LOW-DENSITY GYPSY MOTH (LEPIDOPTERA: LYMANTRIIDAE) POPULATIONS BY MATING DISRUPTION WITH PHEROMONE

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Abstract—This four-year study demonstrated that low-density populations of the gypsy moth, *Lymantria dispar* (L.), were effectively suppressed by annual aerial application of 75 g of racemic disparlure per hectare formulated in plastic laminate flakes. These tests also showed that, when plots were treated with 150 g of pheromone per hectare in 1990 only and left untreated for the following three years, populations continued to be suppressed in 1991–1993 as compared with the controls. Although none of the plots were treated in 1994, population assessment continued and showed that the gypsy moth population density remained low in the plots that had been treated annually for the preceding four years. The laminate flakes released an average of 0.48 g disparlure per day per hectare from each of the two applications in 1990, and 0.72 g per day per hectare from the single application in each of the following three years (1991–1993). Only 27–40% of the applied pheromone dose was emitted during male moth flight.

Key Words—Gypsy moth, *Lymantria dispar*, disparlure, pheromone, mating disruption, laminate flakes, forest pest, aerial application.

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INTRODUCTION

The gypsy moth, *Lymantria dispar* (L.), is a major pest of forests in the eastern United States, Michigan, and parts of Canada. In addition, over the last 10 years, approximately 32 isolated infestations per year have been detected and eradicated in states outside of the generally infested area (McGovern, 1994). Studies on the use of the sex attractant pheromone, (Z)-7,8-epoxy-2-methyloctadecane or disparlure (Bierl et al., 1970), for disruption of mating in low-density populations have been carried out for the past 15–20 years (Cameron, 1981). Tests of aerially applied, controlled-release formulations of disparlure conducted in the late 1970s (Webb et al., 1981) showed that gypsy moth mating was reduced by approximately 70–85% or more depending upon the population density and the applied dose (Schwalbe et al., 1979, 1983). Dose–response studies (Webb et al., 1988) showed that the degree of mating disruption increased as the amounts of disparlure in plastic laminate flakes was increased from 7.5 to 75 g/ha in aerially treated plots.

Ground applications of disparlure at rates of 5, 50, and 500 g/ha, dispensed from plastic laminate tapes in plots with high-density gypsy moth populations, resulted in 6.5, 34, and 84% decreases in mating success, respectively, as measured against controls in small plot tests (Schwalbe and Mastro, 1988). Similar studies with disparlure applied in plastic laminate tape (plastic rope) showed that doses as high as 250 g/ha were insufficient to disrupt mating in isolated, high-density populations in which pretreatment surveys showed that immature stages of gypsy moths averaged over 40 per burlap-banded tree (Webb et al., 1990). A five-year evaluation of the same technology (50 g disparlure/ha applied every other year in plastic laminate tapes) demonstrated biological activity for two seasons as measured by reduced mating success and male moth trap capture. However, these ground treatments were not effective in dampening the rate of population increase as measured by egg-mass densities over the five-year study (Kolodny-Hirsch et al., 1990). Part of the lack of efficacy was attributed to inadequate permeation of the ground-applied disruptant throughout the upper canopy of the trees. This study found a significant increase in mating success of females deployed at a height of 15 m as compared with those placed at a height of 1.5 m. These results suggest that, for mating disruption to be effective, the pheromone must be distributed vertically throughout the canopy and remain there for the duration of adult activity. An aerially applied laminate flake formulation of disparlure resulted in the deposit and adherence of the flakes throughout all strata of the canopy and yielded season-long mating disruption in Giles Country, Virginia (Leonard et al., 1992).

This paper reports the results of a five-year (1990–1994) U.S. Department of Agriculture (USDA) cooperative program within the Forest Service's Appalachian Gypsy Moth Integrated Pest Management Program (AIPM) to disrupt

mating in low-density gypsy moth populations in relatively large woodlots in Virginia. These tests compared plots aerially treated with one application of a laminated plastic flake formulation of disparlure in each of the first four years with plots treated with two applications of the flakes only in 1990 followed by no treatment in succeeding years. Efficacy and formulation release rates were determined for each treatment.

METHODS AND MATERIALS

Treatments. The test sites were isolated woodlots (each 13.8–63.9 ha, Table 1) in Rockbridge County, Virginia, in an area that was newly infested with the gypsy moth. It was considered to be part of the southern leading edge of the generally infested area. In 1989, the year prior to treatment, captures of male moths in USDA milk carton survey traps (2 km spacing) averaged 27 males/trap in the area encompassing the test plots. Nine individual woodlots, each surrounded by agricultural fields, were selected on the basis of this male moth survey data and stand composition (greater than 70% basal area oak, *Quercus* spp.). At the beginning of the study, the nearest dense gypsy moth population was approximately 20 km from any of the test plots. The woodlots were randomly assigned to one of three treatments for these mating disruption studies (Table 1). Three plots, C1, C2, and C3, were untreated controls. Three plots, 1L1, 1L2, and 1L3, were treated annually (1990–1993) with a single application of 75 g disparlure/ha just prior to initial male moth flight. The final three plots, 2L1, 2L2, and 2L3, were treated with two 75 g disparlure/ha applications only in 1990; one treatment was applied just prior to initial male moth flight and the second treatment was prior to peak flight. Treatment dates were selected based on the development of immature life stages and expected male eclosion. Monitoring of male trap catches confirmed that application dates occurred within the desired time period. For 1990 through 1993, initial treatments were applied, respectively, on June 26, 17, 30, and 23. The second treatment of pheromone to plots 2L1, 2L2, and 2L3 in 1990 was applied on July 16. All pheromone treatments were made using specialized equipment (Schwalbe et al., 1983) attached to a Cessna 206 aircraft and calibrated to deliver 75 g active ingredient (a.i.) in 405 g flakes per hectare per application. Two of the nine study plots were no longer available in 1994 due to actions by the property owners; control plot C3, defoliated by high-density gypsy moth populations in 1993, was treated with chemical insecticide and plot 1L3 was clear-cut. None of the remaining plots was treated with pheromone in 1994.

The plastic laminate flakes (1 × 3 mm) had 3-mil PVC outer layers and an inner polymer layer containing racemic disparlure (Disrupt II; Hercon Environmental Co., Emigsville, Pennsylvania). The flakes (18.5% a.i.) were pack-

TABLE 1. DESCRIPTION OF TEST PLOTS AND LIFE-STAGE MONITORING

		Plot description[a]								
	Year	C1	C2	C3	1L1	1L2	1L3	2L1	2L2	2L3
Plot size (ha)	All	63.9	13.8	27.5	36.4	23.1	49.8	57.4	33.2	26.3
Subplots (*N*)[b]	1990	209	34	89	92	86	209	176	142	104
	1991	209	34	89	92	86	209	176	142	104
	1992	206	40	92	94	86	173	188	111	114
	1993	210	40	92	94	86	174	188	123	114
Burlap bands (*N*)	1990	418	64	178	184	172	418	352	284	208
	1991	840	80	178	380	344	800	752	492	464
	1992	844	150	355	380	372	692	752	NA[c]	456
	1993	842	143	359	376	355	696	746	492	453
Pheromone-baited	1990	13	3	6	6	9	17	7	6	10
traps (*N*)	1991	11	4	5	5	6	10	10	8	6
	1992	14	4	5	5	4	8	9	7	6
	1993	13	4	5	5	4	8	9	7	6
Monitor females	1990	392/347	362/296	332/228	420/394	395/353	397/373	305/273	300/240	378/328
placed/	1991	336/304	343/305	344/328	500/440	480/460	480/444	345/269	345/269	346/339
recovered (*N*)	1992	370/302	369/309	370/335	370/319	360/312	370/335	No monitor females		
	1993	480/385	480/421	480/425	480/458	479/407	450/338	No monitor females		

[a] C = control plots; 1L = single application of 75 g pheromone in laminate flakes per ha each year; 2L = two applications of 75 g pheromone in laminate flakes per ha only in 1990 and no further treatment.
[b] Subplots (0.01 ha each) surveyed postseason for egg masses.
[c] Data not available.

aged with 4.7% diatomaceous earth to prevent clogging in the application apparatus. This mixture was blended during application with a sticker (Gelva 1990, Monsanto Corp., St. Louis, Missouri) in a 1 : 1 ratio by volume to provide adhesion to leaf surfaces.

Population Sampling Procedures. Because low-density gypsy moth populations are difficult to characterize using counts of egg masses (Wilson and Fontaine, 1978), several techniques were selected to evaluate the impacts of treatments on populations and to follow the population trends through the course of the study. These included: burlap bands for sampling abundance of immature life stages, pheromone trapping of adult males, mating success of monitor females, and wild egg mass abundance and fertility.

Immature Life Stages. Relative abundance of immature life stages (Table 1) was monitored in the plots using burlap bands (25 cm wide) fastened around host trees approximately 1.4 m above the ground (64–844 bands per plot). Sampled trees were spaced uniformly throughout the plots by selecting host trees closest to points on a 50-m grid. Burlap bands have been shown to be useful for sampling low-density gypsy moth populations (Wallner et al., 1989, 1990). Bands were checked once when gypsy moths were in the late-instar or pupal stages and a second time after adult flight when shed pupal cases and egg masses were present. Type and number of life stages were recorded at each sampling period. Egg masses were collected and held under ambient conditions for at least 30 days; following mechanical dehairing, they were inspected for evidence of embryonation.

Male Moth Trapping. USDA milk carton traps baited with the standard pheromone dispenser were used in all plots (Table 1) to monitor adult male moth flight (Schwalbe and Mastro, 1988). Generally one trap was placed every 6.2 ha (3–17 traps per plot); three traps were placed in the one plot (C2) that was smaller than 18.6 ha. All traps were monitored at least twice during the adult flight period and once after the end of male flight. In addition, one trap per plot was monitored daily to confirm that flight did not occur prior to treatment. Trap data from the control plots were used to determine the duration and peak period of male flight activity. Traps were removed from the plots or covered with plastic on the day of pheromone treatment to prevent contamination from aerially applied flakes.

Mating Success of Monitor Females. To monitor mating success, laboratory-reared females were placed in each plot because few wild females were projected to be available for sampling, particularly in the early years of the study. Normally, 30 one-day-old virgin female moths were placed in each plot three times a week. When sufficient numbers of 1-day-old females were not available, they were supplemented with 2-day-old females. In these circumstances, care was taken to uniformly distribute 1- and 2-day-old females among all plots. Females were shipped as pupae four to eight days after pupation from

increase in counts of immatures compared to the 1993 numbers (Table 2), even if the 1993 data are adjusted to exclude data from the control plot that was treated with insecticide in 1994. Such adjustment gives a mean immature density of 801 life stages/100 bands in 1993 instead of 709.

Counts of immatures under burlap bands in 1994 in the two remaining 1L plots averaged 17.6 life stages/100 bands. This appears to be a decrease in density as compared with the 1993 data (Table 2) even when the 1993 data are adjusted to remove the data for the woodlot lost to clear cutting. The adjusted mean number of life stages/100 bands in 1993 for the 1L plots is 46.4. This suggests that the gypsy moth population did not rebound in 1994, the first year that these plots were not treated with pheromone. In the three woodlots treated only in 1990 (2L), counts of immatures remained relatively low in the two years following treatment (1991 and 1992), but then increased about 10-fold in each of the next two years to a level where they were similar to those found in control plots.

Male Moth Captures. Captures of male moths in all of the plots in the first year of treatment (1990) were relatively low, but the mean male captures in the control plots (33.4 males/trap) was higher than those in either the 1L (0.0 males/trap) or 2L (0.3 males/trap) plots (Table 2). The numbers of male moths captured in the control plots were also higher in each of the following four years than the numbers captured in treated plots and increased to a mean of 1960 males/trap in 1993. In 1994, male captures in control plots (716 males/trap) were lower than the 1993 captures even if data from the control plot that was lost in 1994 (due to pesticide application) are excluded from the 1993 captures; with that adjustment, the two remaining control plots had a mean capture of 1104 males/trap in 1993.

Although gypsy moth populations adjacent to the experimental plots also had become very high, the mean number of males captured per trap in the 1L plots remained less than one per trap in each of the first four years of treatment. In 1994, the first year with no treatment, the number of males captured in the 1L plots was still very low ($\bar{X}$ = 8.5/trap) when compared with the control. Once more this suggested that the populations in the 1L plots remained suppressed, even though treatment ceased in 1993. Similarly, the numbers of males captured per trap in the 2L plots remained low ($\bar{X}$ = 0.5 males/trap) in the first year following treatment (1991), but increased each year thereafter to 534.4 males/trap in 1994 (Table 2). The data analysis showed that numbers of males captured over time differed significantly (P = 0.03) among the treatments.

Mating Success of Monitor Females. In 1990, none of 1961 monitor females recovered from the six treated plots (1L and 2L) was mated, but an average of 10.3% of the 931 females recovered from the control plots were mated. This relatively low mating success in the control plots may be attributed to low male mating pressure before and after peak male emergence. Female placements were

initiated a week prior to anticipated male flight and were continued until no males were trapped in control blocks for three consecutive days. During the two weeks of peak male flight (July 16–30, 1990), the percentage of mated monitor females in the control plots was substantially higher at 17.4% compared to 5.5% before and 4.8% after peak flight. In 1991, there was still no mating of the monitor females in the annually treated plots (1L) and only 0.2% mating (2 of 966 females) in the 2L plots. In contrast, 2.5% of the 937 females placed in the control plots in 1991 were mated, and 4.2% of those placed in the two weeks of peak male flight were mated. In 1992 and 1993, 0.7% and 0.1%, respectively, of the monitor females were mated in the 1L plots as compared with 67.7% and 52.8%, respectively, in the control plots. Data analysis for all three treatments for the first two years (1990 and 1991) showed a significant ($P = 0.004$) treatment × year interaction effect. This indicated that mating success changed differently for each of the three treatments and that these differences were significant. A separate analysis showed that mating success in the control plots over 1990–1993 was significantly ($P = 0.005$) different from that in the 1L plots. Monitor females were not placed in any plots in 1994.

Wild Egg Mass Abundance and Fertility. Analyses of numbers of fertile egg masses under burlap bands ($P = 0.0001$) and numbers of fertile egg masses discovered in surveys of 0.01-ha subplots conducted late in the year ($P = 0.007$) indicated that the density changes over time were significantly different for the three treatments.

Survey of the 0.01-ha subplots prior to treatment in the first year of the test revealed only one egg mass and that in plot C3. Numbers of fertile egg masses found under burlap bands in control plots increased over the first four years of study (Table 2). After the adult flight period in 1990, burlap bands in control plots averaged 3.5 fertile egg masses/100 bands. These numbers rose steadily for the next three years and averaged 594 fertile egg masses/100 bands in 1993. This constitutes a 170-fold increase over four years. Over the same period, the egg mass surveys in the 0.01-ha subplots indicated that an even greater increase in the fertile egg-mass density had occurred in the control plots than was indicated by the burlap-band survey. Mean numbers of fertile egg masses per hectare found in the control plots during these surveys rose from 10.1 in 1990 to a high of 3137 in 1993 (a 310-fold increase). In the control plots, 99% of all of the egg masses found in 1993 under burlap bands or in the postseason surveys were fertile. The 1994 egg mass counts from burlap-banded trees in the control plots apparently decreased even when the 1993 data are adjusted to remove data for the control plot that was lost in 1994 (adjusted 1993 mean is 443.2 egg mass/100 bands). Density estimates from sampling 0.01-ha subplots in control plots also indicated a decrease in egg mass density in 1994 (Table 2); however, when the 1993 data are adjusted to eliminate those from the lost replicate, the 1993 number of fertile egg masses per hectare becomes

2292, which is similar to the 1994 number. The contrast between this information and the 1994 decline in egg mass density indicated from the burlap band counts might suggest that, in low density populations, burlap bands are more sensitive for detecting egg masses than are the standard 0.01-ha subplot surveys. However, in high populations, the burlap bands can become saturated and the counts fail to indicate the true population level.

In plots treated annually from 1990 to 1993 with pheromone (1L), the numbers of fertile egg masses found under the burlap bands remained low throughout the years of treatment (Table 2). Although the mean number of fertile egg masses in the 1L plots was 3.9/100 bands in 1993, more egg masses (mean of 13.4/100 bands; data not shown) were actually found but only 29% of these were fertile. Postseason egg mass sampling in 0.01-ha subplots showed low numbers of fertile egg masses per hectare in 1990–1992 in the 1L plots but, in 1993, this number increased to 13.8 fertile egg masses per hectare reflecting the pressure from the surrounding high-population areas (Table 2). In 1994, the first year in which these 1L plots were not treated, the number of fertile egg masses under burlap bands and in the 0.01-ha subplots remained similar to the 1993 levels (Table 2). This assessment does not change markedly when the data for the replicate that was lost in 1994 are eliminated from the calculation of the 1993 means. The adjusted numbers of fertile egg masses are 5.3/100 bands and 18.7/ha for 1993, and these means remain comparable to the numbers obtained in 1994. As with other population measures, the fertile egg mass data suggest that the gypsy moth populations remained low in the year after the treatment was terminated.

In plots treated only in 1990 (2L), the mean number of fertile egg masses per 100 bands was very low (0.0) in the year of treatment as well as in the year following treatment (0.3), but rose thereafter (Table 2). Similarly, the mean number of fertilized egg masses per hectare found in the 0.01-ha surveys on the 2L plots were 0.0 and 0.3 in 1990 and 1991, respectively, but rose to 9.1 in 1992 and eventually to 1268 in 1994. Counts under burlap bands as well as in the 0.01-ha surveys increased approximately fourfold between 1993 and 1994 (Table 2). It appears that populations in these plots that had only been treated in 1990 were rapidly approaching densities observed in control plots.

Release Rates. Results of analyses for residual disparlure content in flakes were used to generate linear regressions of residual content with the square root of aging time (Figure 1). The rate at which disparlure was delivered by the flakes declined over the period of male moth flight (about six weeks). For each of the 1990 applications, the average release rate over the first six weeks of the test, calculated from the regression equations, was 0.94 μg/day/flake with mean rates during the first and sixth week of 2.31 and 0.49 μg/day/flake, respectively. Flakes picked from foliage gave similar values as those from spray cards (data not shown). For the 1991–1993 applications, the calculated average rate was

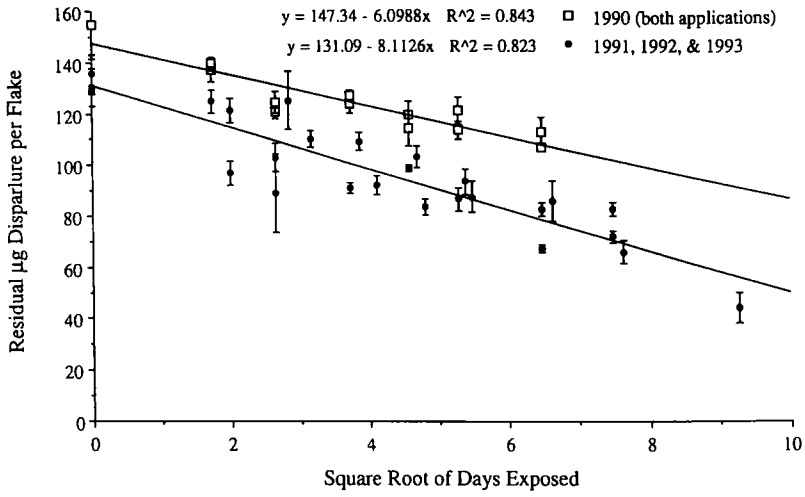

FIG. 1. Residual quantity of disparlure in flakes retrieved from spray cards in 1990–1993 field tests in Rockbridge County, Virginia. Linear regression of disparlure content with square root of time (days) exposed. Error bars show standard errors of sample means.

1.25 µg/day/flake over the first six weeks with rates during the first and sixth weeks of 3.07 and 0.53 µg/day/flake, respectively.

The release rates were used to calculate the amount of disparlure delivered per day per hectare over the course of male moth flight (Figure 2). Since the 2L plots received two applications in 1990, the additive effect is shown in Figure 2A. Each of the two 1990 applications delivered a total of 20.1 g of disparlure over the first six weeks after application for an average rate of 0.48 g/day/ha per application; this is 27% of the applied 75-g dose. The 1991–1993 applications (Figure 2B) each released 30.1 g or 40% of the applied pheromone per hectare over the first six weeks of the test for an average rate of 0.72 g/day/ha. Since male moth flight in Rockbridge County Virginia virtually ceases by the end of July, pheromone released from the flakes six weeks after mid-June application does not contribute to the treatment impact and is essentially wasted. Based on these release rates, about 60–73% of the applied pheromone in the laminate flakes was not released during moth flight in the year of application.

Analysis of flakes retrieved a year later from the 1990 application showed that 2.5 µg/flake of disparlure or about 1.8% of the original dose remained after 12 months of exposure in the field. Therefore, during moth flight in 1991, a

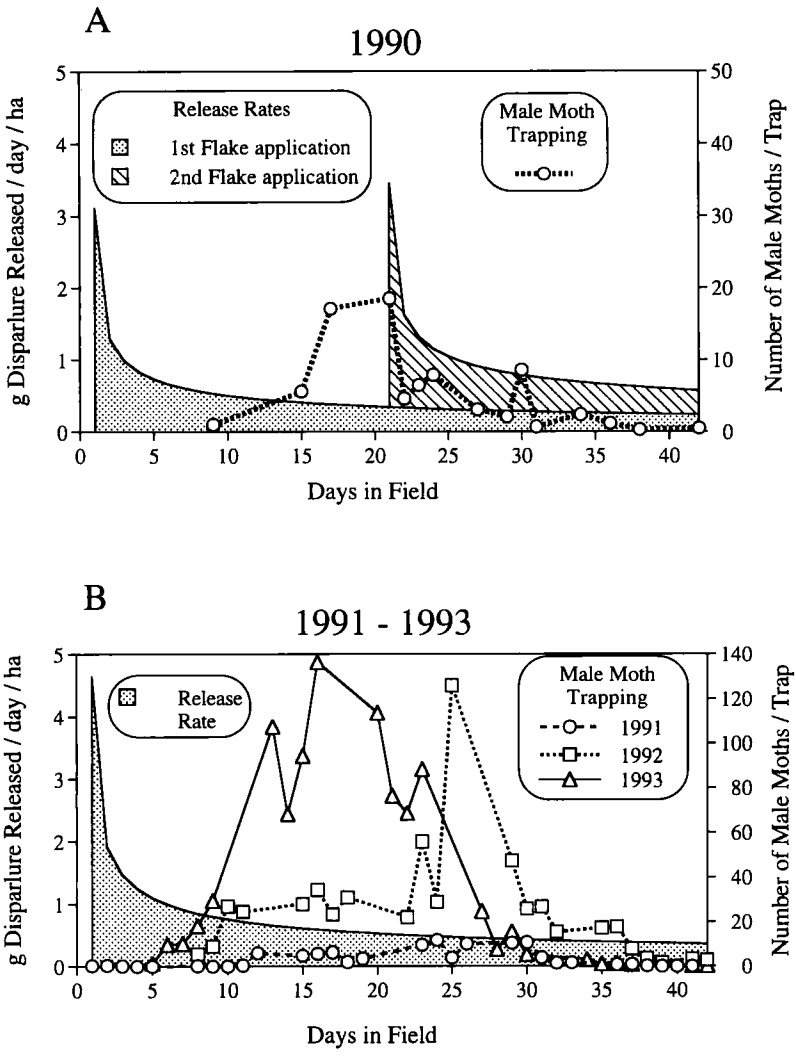

FIG. 2. Amount of disparlure delivered per day per hectare over the course of male moth flight. (A) Single and double applications in 1990. (B) Annual single applications in 1991–1993. Times of male moth flight relative to pheromone application dates are illustrated by overlayed line graphs of trap captures in control plots.

small amount of disparlure remained in the 2L plots that had been treated only in 1990.

DISCUSSION

Previous mating disruption studies with the gypsy moth have attempted to show an impact on populations only in the year of treatment. They often demonstrated that male moth captures in traps could be reduced or entirely eliminated and that mating success could be reduced, although seldom eliminated, in plots treated with pheromone. In this study, an attempt was made to study the longer-term impacts on population trends from a single treatment and from yearly treatments. The results of this study demonstrated that population densities were impacted by either type of treatment. Compared to control plots, the abundance of immature life stages and fertilized egg masses, the percentage of mating success of monitor females, and the number of trapped adult males all remained low in plots treated yearly with one application of 75 g disparlure/ha (1L plots). The reduction of monitor female and wild female mating successes indicated there was significant mating disruption in these treated plots in all four years of treatment. Although monitor females were not deployed in these plots in 1994, the year following the last pheromone treatment, the measures of population density showed that the gypsy moth populations remained suppressed.

In the 2L plots, all measures of population density were low in the year of treatment (1990) as well as in the succeeding year (1991). However, in 1992, all measures indicated an upward trend in population density. Nevertheless, it appears that the double application of pheromone in these 2L plots in 1990 resulted in populations that were lower than those in the control plots in 1992 and 1993. Meanwhile, surrounding populations and populations in control plots increased rapidly and eventually reached defoliating levels. Although the woodlots chosen for this study provide some degree of isolation from migrant males and perhaps from ballooning first-instar larvae (McManus, 1973), these plots are not totally isolated. Therefore, it is surprising (and encouraging) that the 2L plots treated only in 1990 continued to show depressed populations for one to three years as compared with the control plots.

In past studies, the absence of trap captures of males has not been equated with the absence of female mating success. It has been speculated that females are more attractive because of chemical or visual cues. Visual cues have been shown (Richerson et al., 1976; Richerson, 1977; Charlton and Cardé, 1990) to be important in male close-range orientation. However, more time is generally required for a male moth to enter a trap than to find an exposed female on a tree bole (Mastro et al., 1977). The longer the required searching time, the higher the probability that a male will discontinue the search and move on. In

this study, the monitor females were placed in a sheltered location more like native female sites in a low-density gypsy moth population. However, the shelter did not expose the females and required males to spend more time searching. While mating success of these females may have been greater if they had been exposed, it has recently been shown that mating success in low-density populations is normally low (Sharov et al., 1995).

Results of this study indicate that low-density gypsy moth populations can be substantially suppressed with annual application of 75 g disparlure/ha. It is yet to be determined what population level (as indicated by egg-mass surveys, numbers of immatures under burlap bands, or male moth trapping) can be targeted for control by the mating disruption technique. The data in this study suggest that, in the year of pheromone application in treated plots, male moth captures may be somewhat unreliable for determining the level of mating success. For example, male trap captures were low in the 1L plots in 1993 (0.6 males/trap), yet an average of 3.9 fertile egg masses/100 bands and 13.8 fertile egg masses/ha were found. Even low male captures in traps may act as a warning that a treatment did not entirely eliminate mating. Based on the four years of this study, it appears that if a pretreatment survey on 0.01-ha subplots shows less than two fertile egg masses per hectare, and if the number of immatures (larvae + pupae) per 100 burlap bands averages approximately five or less, it is highly likely that application of pheromone will effectively control female mating.

Male moths are highly mobile and attempts to relate trap captures to other measures of population density have not been successful under all conditions (Liebhold et al., 1995). Therefore, the mean numbers of immatures and fertile egg masses under burlap bands and the numbers of fertile egg masses found in late-season 0.01-ha subplot surveys were used to obtain a population index for all nine plots during the five years of this study. The sum of the means of these three population measures (immatures per 100 bands, fertile egg masses per 100 bands, and fertile egg masses per hectare) were plotted versus year (Figure 3) to indicate the population changes over time for the three treatments. From this graph, it is apparent that the single pheromone application in 1990 (2L plots) delayed the population increase by one to four years. Annual pheromone application controlled the population for three years and slowed the rate of increase thereafter.

SUMMARY

This study showed that the population represented by an average male moth capture of 27/trap in the vicinity of the test plots in 1989 was effectively controlled for at least three years by annual pheromone application until neighbor-

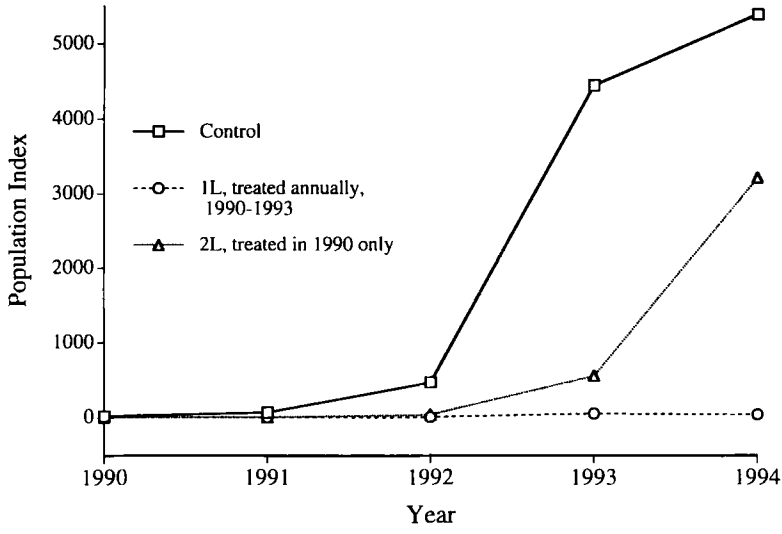

FIG. 3. Population index for all nine study plots (sum of mean number of immatures/100 bands + mean number of fertile egg masses/100 bands + mean number of fertile egg masses/ha) plotted by treatment for each of the five years of the study.

ing, uncontrolled populations exerted too high an influence. However, despite this population pressure from surrounding areas, the single application of 75 g of pheromone/per hectare significantly disrupted mating in each of the four treatment years. This study also showed that treatment with pheromone in only one year (albeit a double application) substantially suppressed the population increase as compared with the controls for three subsequent years. Thus, in properly selected populations, mating disruption with aerially applied pheromone is an effective control method.

The release profile of disparlure from the laminate flakes is characteristic of a laminated structure (Kydonieus, 1980) and has the advantage that exact timing of the application just before male flight is not critical. However, the fact that only 27–40% of the applied pheromone was released during male flight suggests that a controlled-release formulation, which more efficiently delivers its pheromone dose, would allow the total amount of applied pheromone to be reduced by perhaps a factor of two without loss of efficacy. Such a formulation would reduce treatment costs. Work is now being carried out to develop such a delivery system.

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A NEW ASSAY FOR QUANTIFYING BROWN ALGAL PHLOROTANNINS AND COMPARISONS TO PREVIOUS METHODS

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Abstract—Quantitative measurement of phlorotannins (polyphenolics) in brown algae (Phaeophyta) by colorimetric assays can be confounded because: (1) most such assays also react to nonphlorotannin substances (interferences) and (2) the appropriate reference compound for such assays is not always clear, although phloroglucinol is typically used. We developed a new assay in which 2,4-dimethoxybenzaldehyde (DMBA) reacts specifically with 1,3- and 1,3,5-substituted phenols (e.g., phlorotannins) to form a colored product. This new assay, as well as eliminating the problem of measuring interferences, is inexpensive, rapid, and can be used with small sample volumes. We recommend it for all assays of phlorotannins from one or a set of closely related species where the structural types of phlorotannins present are likely to be similar among samples. It is also appropriate for broader surveys of phlorotannin levels across many species, but in this case a reference must be chosen with care. We also compared the DMBA assay to existing assays, including the Folin-Denis [both before and after the samples were mixed with polyvinylpolypyrrolidone (PVPP)] and the Prussian blue assays. PVPP was not 100% efficient (and often much less) at removing phlorotannins from solution, and its effectiveness varied among different phlorotannins. Thus, in contrast to previous studies, measuring phenolic levels in extracts before and after

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treatment with PVPP will not necessarily result in an interference-free measure of phlorotannins. Based on an analysis of reactive substances in red and green algae (which do not contain phlorotannins) in the Folin-Denis and Prussian blue assays, we estimate that the average level of interferences (nonphlorotannins) in brown algae measured in these two assays is on the order of 0.5% by dry weight.

Key Words—Phlorotannins, DMBA, Folin-Denis assay, brown algae, seaweeds, phenolics, PVPP.

INTRODUCTION

Polyphenolics are one of the most common classes of secondary metabolites in terrestrial plants (Harborne, 1991; Haslam, 1989; Swain, 1979), marine angiosperms (McMillan, 1984), and macroalgae (Ragan and Glombitza, 1986; Steinberg and van Altena, 1992). They are ecologically important in both terrestrial and marine systems, deterring or inhibiting herbivores, epiphytes, pathogens, or competitors (e.g., allelopathy) (Beress et al., 1993; Bernays et al., 1989; Hay and Fenical, 1988). In particular, there is a substantial empirical and theoretical literature on the effects of polyphenolics against herbivores (Coley et al., 1985; Feeny, 1976; Steinberg, 1992; Swain, 1979). Polyphenolics can also significantly affect broad-scale ecosystem properties in both terrestrial (Schlesinger, 1991) and marine (Carlson and Mayer, 1983) systems.

Although terrestrial and marine plant polyphenolics are similar in some respects, there are some fundamental differences among the compounds. Terrestrial polyphenolics, or tannins, are polymers based on flavonoids or gallic acid (Haslam, 1989). Algal polyphenolics, or "phlorotannins" (Ragan and Glombitza 1986), which are only known from the brown algae (Phaeophyta), are restricted to polymers of phloroglucinol (1,3,5-trihydroxybenzene). Phlorotannins and terrestrial tannins vary in their linkages between monomeric units, in their size (dimers to 600,000 amu), and to some extent in the types of substitutions present (i.e., halogenation, sulfated groups) (Ragan and Glombitza, 1986).

While the abundance and ecological importance of polyphenolics in natural systems has long been recognized, the ability to quantitatively measure these compounds in plant tissue remains a complex issue (Mole and Waterman, 1987a,b; Ragan and Glombitza, 1986; Ragan and Jensen, 1977; Van Alstyne, 1995). Because the compounds are typically large, polar, and water soluble, they are difficult to measure by the standard analytical techniques used to analyze smaller nonpolar metabolites such as terpenes and acetogenins. Analysis of algal polyphenolics is further complicated by the difficulty of separating the individual compounds from the polymeric mixtures which occur naturally in vivo.

Consequently, polyphenolics have typically been analyzed in one of two

ways. The first is via various spectrophotometric procedures based on the reaction of a colored reagent with the easily oxidized phenolic functional group. The best known of these procedures is the Folin-Denis test, or variations thereof, such as the micro-Folin Denis (Arnold et al., 1995; Targett et al., 1995; Van Alstyne, 1995) or Folin-Ciocalteau (1927) tests. This assay and its variations have been used extensively in the analysis of phenolics from both marine and terrestrial plants (Ragan and Jensen, 1977; Mole and Waterman, 1987a,b; Steinberg, 1989). The second set of methods uses the ability of polyphenolics to bind to macromolecules such as proteins, thus generating a measure of "relative tanning ability" (Hagerman and Butler, 1989).

Such techniques have been very useful, but they are subject to a number of sources of error, of which two are perhaps the most significant for phlorotannins (Ragan and Glombitza, 1986; Steinberg, 1989; Van Alstyne, 1995). Firstly, because specific molecules are not analyzed, but rather total phenols or tanning ability, variation in the reactivity of structurally different polyphenolics (due to variation in size, structure, etc.) is not separable from variation in reactivity due to different amounts of compounds. Secondly, nonpolyphenolic interferences can react with the reagents used in some colorimetric assays, resulting in an overestimate of phenolic levels.

In this paper we compared three spectrophotometric assays in an attempt to assess the variation in the three assays resulting from different types of phlorotannins from marine brown algae and to determine the effect of interferences in these assays. We compared the Folin-Denis technique, used with and without polyvinylpolypyrrolidone (PVPP), a resin which binds to, and removes polyphenols in solution; the Prussian blue assay, which has been suggested to be less sensitive than the Folin-Denis assay to interfering compounds; and a new assay, which is based on the ability of 2,4-dimethoxybenzaldehyde (DMBA) to react specifically with 1,3- and 1,3,5-substituted phenols (i.e., phlorotannins), and which does not react with tannic acid containing only ortho- and parahydroxyl-substituted phenolics). We compared the color yield of these three techniques for both purified phlorotannin fractions and crude extracts from a number of brown algae. The effect of interferences on the measurement of phlorotannins was examined by measuring phenolic levels in our extracts before and after the addition of PVPP and by measuring the reactivity of extracts from red and green algae, which lack phlorotannins.

METHODS AND MATERIALS

Chemical Assays

Phenolic levels in crude extracts of brown algae and in "purified" phlorotannin fractions (see Preparation of Pure Phlorotannin Fractions, below) were

quantified by three methods. The first, the Folin-Denis procedure for measuring total phenolic content (Ragan and Jensen, 1977; Swain and Hillis, 1959), has been used extensively for measuring phlorotannins in brown algae (Ragan and Glombitza, 1986; Steinberg, 1989; Targett et al., 1992; Van Alstyne, 1995). The second, the Prussian blue assay (Price and Butler, 1977), is also well described in the literature but has not been used as extensively as the Folin-Denis procedure. Our new technique, the DMBA assay, is based on the vanillin–H_2SO_4 reaction (Butler, 1982; Butler et al., 1982; Putnam and Butler, 1985).

Folin-Denis Assay. Folin-Denis reagent was prepared by dissolving 25 g of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) and 5 g of dodecamolybdophosphoric acid ($12MoO_3 \cdot H_3PO_4 \cdot H_2O$) in 175 ml distilled water, adding 12.5 ml phosphoric acid to the solution, boiling under reflux for 2 hr, and then making up to 250.0 ml. The sample (1.0 ml) was mixed with 4.0 ml of this reagent and 8.0 ml of a saturated sodium carbonate solution and then made up to a reaction volume of 25.0 ml with distilled water. Color was allowed to develop for 5–6 hr, which allowed the precipitate that formed to settle before the absorbance was read at 725 nm.

Prussian Blue Assay. Samples were comprised of 100 μl of extract or diluted extract in 50.0 ml of distilled water in a 125 ml flask. Three milliliters of ferric ammonium sulfate (0.1 M $FeNH_4(SO_4)_2$ in 0.1 M HCl) was then added to successive samples at intervals of 1.0 minute. Exactly 20 min after the addition of ferric ammonium sulfate to each sample, 3.0 ml of potassium ferricyanide [0.008 M $K_3Fe(CN)_6$] was added. Exactly 20 min after the addition of potassium ferricyanide, the absorbance at 720 nm was read. Solvent-only blanks were included and subtracted from sample absorbances. Like Folin-Denis, Prussian blue reacts with reducing compounds such as phenols, but the phenolic group is not directly incorporated into the colored product.

DMBA Assay. Stock solutions of 2,4-dimethoxybenzaldehyde (2 g/100 ml solution) (Sigma D-3269) and of hydrochloric acid (16.0 ml concentrated hydrochloric acid per 100.0 ml solution) were prepared in glacial acetic acid. The working reagent, prepared by mixing equal volumes of these two solutions just prior to use, was kept at room temperature before starting the assay. The working reagent is slightly yellow when mixed, but becomes purple (λ_{max} = 565 nm) upon standing, so it must be prepared fresh each day. Blanks of the same volume as the sample were used in all assays because some color formation occurs in the absence of phlorotannins.

To ensure that the response was linear for each type of purified tannin, standard curves were prepared. Aliquots of each phlorotannin solution (up to 40 μg from 10 μg/μl solution) were mixed with 10 μl of DMF (*N*,*N*-dimethylformamide) and 2.5 ml of the working reagent (DMF is necessary for use in protein precipitation assays as described in Stern et al. (in press). The reaction mixtures varied in volume by 4 μl (of 2.5 ml) when testing different concentra-

tions; this was done to minimize the volume of added methanol, at the lowest level of pipetting possible (1 μl), within the range of sensitivity of the assay (10 μg). The absorbance was determined at 510 nm after exactly 60 minutes at 30°C. For fresh extracts of brown algae, 10–100 μl were used in the DMBA assay, depending on the concentration of phlorotannins.

Preparation of Pure Phlorotannin Fractions. In order to assess the variation in response of different phlorotannins in the different assays, phlorotannin fractions from six species of brown algae were prepared following the methods of Ragan and Glombitza (1986) as modified by Steinberg and van Altena (1992) and van Altena and Steinberg (1992). In brief, several kilograms of fresh seaweed were extracted in 80% methanol under nitrogen at 4°C for three days with regular mixing. The filtered extract was rotary evaporated to a small aqueous volume at 40°C. Any precipitates were then filtered and the filtrate was lyophilized, triturated with methanol several times (to remove salt), and then adsorbed onto microcrystalline cellulose. This column was eluted with toluene (to remove pigments) and then with ≈3 liters of 2:1 acetone–water. This was rotary evaporated, redissolved in water, and then freeze dried. These phlorotannin fractions contained a mixture of phlorotannin polymers and oligomers but were pure in the sense of containing 95+% phlorotannins, based on the absence of peaks in ^{13}C NMR spectra except those in the three regions typical of phlorotannins (96–106, 124–131, and 141–164 ppm) (van Altena and Steinberg, 1992).

Phlorotannins from the kelps (Order Laminariales) *Agarum cribrosum*, *Ecklonia radiata*, and *Dictyoneurum californicum*, and from the fucoids (Order Fucales) *Sargassum vestitum* and *Carpophyllum maschalocarpum* were used. High-molecular-weight phlorotannins from the fucoid *Fucus vesiculosus* were obtained from M. A. Ragan. Catechin (C1251; Sigma, St. Louis, Missouri) and phloroglucinol (1,3,5-trihydroxybenzene) (P3502; Sigma) were also tested in the assays.

Assays of Phenolic Levels in Fresh Extracts of Brown Algae. Phenolic levels in fresh methanol extracts of a variety of brown algae also were measured. Extracts were prepared by grinding fresh algal tissue (0.5–1 g) in a Sorvall Omni Mixer in 85% MeOH and storing at 4°C overnight prior to assaying for phenolic levels.

Extracts from 11 species of Australian brown algae (Phaeophyta) from four orders were measured using the DMBA and Folin-Denis assays. These included representatives of the Order Laminariales (*Ecklonia radiata*), Dictyotales (*Dilophus marginatus*, *Dictyopteris acrostichoides*, *Dictyota dichotoma*, *Homoeostrichus sinclarii*, *Zonaria diesingiana*), Ectocarpales (*Endarachne binghamiae*), and Fucales (*Sargassum vestitum*, *S. linearifolium*, *Phyllospora comosa*, *Cystophora moniliformis*). For each species, six individual algal plants were collected in summer (February and March) in New South Wales, Australia.

In New Zealand, nine species of brown algae were assayed by the Prussian

blue and Folin-Denis techniques for comparison of relative sensitivity to interferences. These included *Carpophyllum flexuosum*, *C. maschalocarpum*, *C. angustifolia*, *C. plumosum*, *Ecklonia radiata*, *Sargassum sinclarii*, *Xiphophora chondrophylla*, *Lessonia variegata*, and *Cystophora torulosa*. Five individuals from each species were collected and analyzed in summer and autumn at the University of Auckland, Leigh Marine Laboratories, New Zealand.

Assessment of Interferences

Phenolic levels in plant tissue may be overestimated if nonphenolic compounds in algal extracts react with assay reagents, e.g., ascorbic acid or peptides in the Folin-Denis assay (Ragan and Glombitza, 1986). While the DMBA assay was developed in order to avoid such interferences, this assay may not be suitable for all studies. Thus, the problem of interferences was further addressed in two ways.

PVPP Treatments. Polyvinylpolypyrrolidone (PVPP) is a polymer that can remove tannins and phlorotannins from acidic aqueous solution (Loomis and Bataille, 1966; Yates and Peckol, 1993). Thus, one way of assessing the levels of interferences in an algal extract is to measure total phenolics in the extract with the Folin-Denis or Prussian blue procedure before and after treatment with PVPP (e.g., Yates and Peckol, 1993). Any color yield in a Folin-Denis assay after treatment with PVPP should be due to nonphlorotannins, such as proteins or ascorbic acid (or free phloroglucinol, which adheres poorly or not at all to PVPP), and in theory an interference-free measure of phlorotannins could then be obtained by subtraction. Extracts treated with PVPP should not yield any color in the DMBA assay, since DMBA reacts specifically with phlorotannins.

We compared phenolic levels in untreated samples and PVPP-treated samples using both the DMBA and Folin-Denis assays. These experiments give us information about both the efficacy of PVPP in removing phlorotannins from solution and the amounts of nonphlorotannin interferences in our fresh algal extracts. Because both Yates and Peckol (1993) and Targett et al. (1995) note that PVPP is effective in a slightly acidic environment, the pH of the methanol extracts of algae was adjusted if necessary by the addition of 10 μl of acetic acid to a pH of ≈ 4. Both fresh extracts and pure phlorotannins were treated with 20 mg PVPP/ml solution (Kellem et al., 1992; Yates and Peckol, 1993). The mixtures were shaken overnight at 4°C, centrifuged, and the supernatants assayed for phenolic level.

"Phenolic" Analyses of Red and Green Algae. A second way of assessing likely levels of interfering compounds in brown algae is to assay species of red and green algae, which lack phlorotannins (Ragan and Glombitza, 1986). Color obtained in such assays should be due only to interferences such as ascorbic acid, polypeptides, or, in the case of some red algae, simple phenols (Phillips

and Towers, 1982). This method assumes that the levels of these interferences, such as ascorbic acid or proteins, are not substantially different in red and green versus brown algae. In our view this assumption would be strengthened by looking at a taxonomically wide range of species. Thus we measured total phenolics in extracts of 12 species of red algae (seven from California, five from New Zealand) and five species of green algae (three from California, two from New Zealand) in the Folin-Denis and Prussian blue assays. The California algae tested were *Prionotus lanceolata*, *Endocladia muricata* (Order Cryptonemiales), *Halosaccion glandiforme* (Rhodymeniales), *Iridaea flaccida*, *Mastocarpus papillata* (Gigartinales), *Porphyra* sp. (Bangiales), *Rhodomela larix* (Ceramiales), *Chaetomorpha* sp., *Cladophora* sp. (Cladophorales), and *Ulva* sp. (Ulvales); New Zealand algae tested were *Vidalia colensoi* (Ceramiales), *Gigartina circiuta*, *Melanthalia abscissa* (Gigartinales), *Prilonia* sp. (Nemalionales), *Pterocladia lucida* (Gelidiales): *Ulva* sp. (Ulvales), and *Codium fragile* (Siphonales). Sample size was five individuals from each species in all cases. Extraction procedures followed those described above for brown algae.

Although the chemistry of the DMBA reaction should preclude any color formation with red and green algae (unless phlorotannins are present), we assayed a selection of red and green algae (which are known to contain secondary metabolites) with DMBA. We extracted and assayed the following seaweeds: *Laurencia rigida*, *Delisea pulchra*, *Caulerpa scalpeliformis*, and *Codium fragile*. Four species that lack known secondary chemistry were also tested (the red algae *Soliera* sp., *Amphiroa* sp., *Corallina* sp., and the green alga *Ulva lactuca*).

RESULTS

Reaction between Phlorotannins and 2,4-Dimethoxybenzaldehyde (DMBA)

Preliminary characterization of the reaction of *Ecklonia radiata* and *Carpophyllum maschalocarpum* phlorotannins with DMBA in glacial acetic acid showed that formation of the colored adduct was both time- and temperature-dependent (Figure 1). A reaction time of 1 hr was selected since at that time absorbance was relatively stable; slight variations in reaction time did not cause large changes in absorbance. Adequate sensitivity was obtained even at room temperature, but we chose to use slightly elevated temperatures (30°C) to ensure good temperature control despite seasonal fluctuations in room temperature. Higher temperatures gave increased sensitivity, but also increased problems with degradation of DMBA and with acetic acid fumes during the reaction. Based on these results, phlorotannins were routinely allowed to react with the DMBA for exactly 60 min at 30°C before determinations were made. The λ_{max} for the colored product of the reaction is 510 nm so spectrophotometric measurements were made at that wavelength.

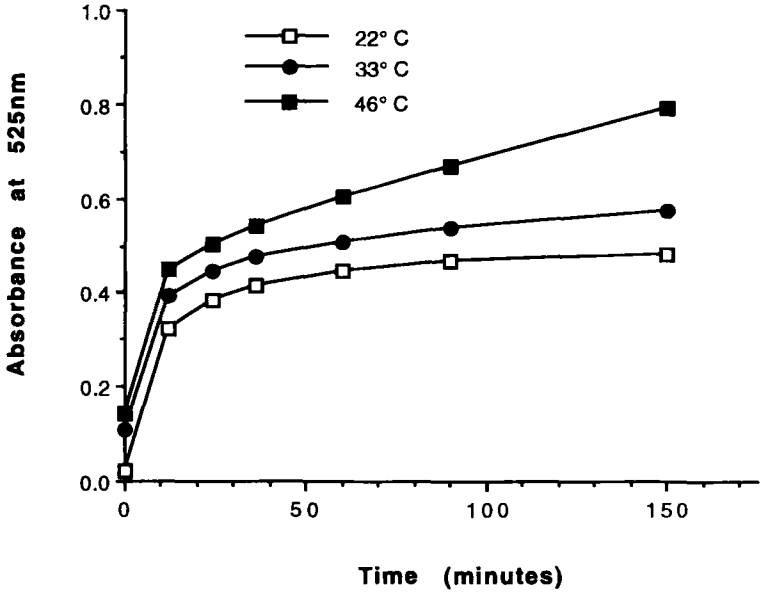

FIG. 1. Kinetics of the reaction between 20 μg of *Carpophyllum maschalocarpum* phlorotannins and 2,4-dimethoxybenzldehyde at three temperatures.

The relationship between mass of phlorotannin and color yield in the DMBA assay for six purified phlorotannin fractions and phloroglucinol is shown in Table 1. The relationship was linear to at least 40 μg, with r^2 values >0.975 for all phlorotannins and phloroglucinol. The limit of detection was approximately 5 μg. The spectra of the reaction products of several different algal phlorotannins with DMBA were similar, indicating that the adduct formed between DMBA and various phlorotannins was the same. Phloroglucinol, the monomer unit of phlorotannins, forms a peak at 510 nm after an initial peak (within the first minute) at 565 nm, which later is masked by the peak at 510 nm. This is probably due to slight differences in the chemistry of phloroglucinol from phlorotannins; e.g., phloroglucinol lacks the aryl and ether linkages of the polymeric phlorotannins (K. Barrow, personal communication).

The DMBA assay is based on chemistry similar to the vanillin assay (Ribéreau-Gayon, 1972). In the vanillin assay, the use of acidified glacial acetic acid as a solvent increases color yield, simplifies the reaction kinetics, and stabilizes the colored product of the reaction between the aldehyde and phenolic (Butler et al., 1982). Furthermore we found that glacial acetic acid is an excellent solvent for phlorotannin/protein precipitates (Stern et al., in press). We suggest

TABLE 1. REGRESSION EQUATIONS FOR ABSORBANCE AS A FUNCTION OF MASS OF PHLOROTANNINS IN THREE DIFFERENT PHENOLIC ASSAYS[a]

Algae	Folin-Denis	Prussian blue	DMBA	$R_{DMBA/FD}$
Phloroglucinol	$y = 0.00290x - 0.0335$	$y = 0.111x + 0.0793$	$y = 0.0620x + 0.053$	1
Agarum cribrosum (L)	$y = 0.00223x - 0.015$	$y = 0.122x + 0.10416$	$y = 0.0140x - 0.020$	0.293
Ecklonia radiata (L)	$y = 0.0020x - 0.017$	$y = 0.0898x + 0.0804$	$y = 0.0190x - 0.006$	0.444
Dictyoneurum californicum (L)	$y = 0.0017x - 0.016$	$y = 0.0920x + 0.0144$	$y = 0.0110x - 0.005$	0.302
Fucus vesiculosus (F)	$y = 0.00256x - 0.007$	not done	$y = 0.0197x + 0.009$	0.360
Carpophyllum maschalocarpum (F)	$y = 0.0013x - 0.013$	$y = 0.0822x + 0.0804$	$y = 0.00640x + 0.002$	0.230
Sargassum vestitum (F)	$y = 0.00083x - 0.087$	not done	$y = 0.00540x - 0.003$	0.304
Overall regression	$y = 0.00207x - 0.0134$	not done	$y = 0.0134x - 0.004$	not done

[a] Equations are in the form: Absorbance = m (μg phenolics) + absorbance. $N = 3$ for each extract at each mass; four masses assayed. The range of masses used were 0–400 μg for the Folin-Denis and Prussian Blue assays, 0–40 μg for the DMBA assay. All r^2 values are >0.975. The overall regression was calculated from data for all compounds. F = Order Fucales, L = Laminariales (kelps). $R_{DMBA/FD} = (m_{DMBA}/m_{PG})/(m_{FD}/m_{PG})$, where m = the slope for standard curves of a given phlorotannin or phloroglucinol (PG); this ratio has been suggested to provide information about molecular weight (Ragan and Glombitza 1986).

the use of a fume cupboard, covered waterbath, and cuvettes with stoppers when using this solvent.

It has previously been reported that small amounts of methanol inhibit the reaction between vanillin and phenolics (Butler et al., 1982). Similarly, we found that as little as 4% methanol, water, or dimethylformamide in the reaction reduced the color yield in the DMBA reaction. When using methanolic or aqueous extracts of plant material, controls must be included to account for this variation. Quantification of brown algal phlorotannins in aqueous methanol extracts (e.g., such as those used here) does not suffer significantly from this decrease in color yield as only very small volumes of extract are required for use in the assay (see Methods and Materials).

Comparisons among Assays Using "Purified" Phlorotannin Fractions and Phloroglucinol

There was considerable variability in the reactivity of different purified phlorotannins within the three assays performed, as indicated in Table 1 by the variation in slopes of the regressions for absorbance versus the mass of each phlorotannin. In general, variation in the reactivity of different phlorotannins was least in the Prussian blue assay, with slopes varying between 0.0822 and 0.111 (including phloroglucinol). Variability in reactivity was greatest in the DMBA assay with slopes varying between 0.005 and 0.02 for the six phlorotannins tested (Table 1, excluding phloroglucinol). The reactivity of phloroglucinol (PG) with DMBA is very high, relative to phlorotannins, with the slope of the standard curve for phloroglucinol in the DMBA assay 3–11 times greater than for the phlorotannin regressions. In comparison, the slope for phloroglucinol in the Folin-Denis assay was 1–3 times higher than slopes for the phlorotannins.

For a given polyphenolic (p), the ratio of color yield in a vanillin assay (V) to a Folin-Denis assay (FD), relative to phloroglucinol (PG) (i.e., $(m_{Vp}/m_{VPG})/(m_{FDp}/m_{FDPG})$ where m is the slope for a given phlorotannin standard curve), may provide information about phlorotannin molecular weight (Ragan and Glombitza, 1986). This is based on the idea that polymerization of phloroglucinol is generally via ether linkages, decreasing Folin-Denis reactive sites (and thus color yield), while vanillin and DMBA color yield is a function of available positions on a benzene ring between meta-oxygen substitutions (Ribéreau-Gayon, 1972). Such positions would not be consumed if polymerization is via ether linkages, and therefore color yield should be strictly proportional to the number of phloroglucinol molecules present. Thus, with increasing molecular weight, vanillin should yield proportionally more color per unit mass than Folin-Denis. Color yield within an assay for a phlorotannin must be taken as relative to color yield for phloroglucinol to account for between-assay differences in extinction coefficients.

Because of the similarity in the chemistry of the vanillin–phlorotannin and DMBA–phlorotannin reaction, we might expect a similar trend for the ratio between DMBA absorbance and vanillin absorbance (vanillin is 3-methoxy-4-hydroxy benzaldehyde). Comparison of these ratios in Table 1 indicates a high ratio for the monomer phloroglucinol, as expected, and the next highest ratios are for the relatively small polymers from *Ecklonia radiata* (van Altena and Steinberg, 1992). However, the polymers from *Fucus vesiculosus*, which span a large size range (Ragan 1985; Ragan and Glombitza, 1986), also yield a high value, suggesting that the ratio of DMBA to FD does not consistently provide easily interpretable information about the molecular weight of phlorotannins (see Discussion, below).

There was some similarity in the response of pure phlorotannins from taxonomically related algae in these assays (Table 1). For example, in the DMBA assay, slopes of the regression lines for the three kelps (Order Laminariales) varied between 0.01 and 0.02 (Table 1). Phlorotannins from the confamilial species *Sargassum vestitum* and *Carpophyllum maschalocarpum* (Order Fucales, family Sargassaceae), had lower slopes: 0.0054 and 0.0064, respectively. A similar grouping was observed in the Folin-Denis assay. However, absorbance of phlorotannins from the fucoid *Fucus vesiculosus* (family Fucaceae) was more similar to that of the kelps in both assays.

Assays of Fresh Algae

The effect of the choice of standard for the determinations of phenolic levels in crude extracts from 11 species of Australian brown algae is shown in Table 2. Phlorotannin levels were measured in the Folin-Denis and the DMBA assay based on three standards: (1) phloroglucinol, the typical standard used in such studies; (2) the overall regression for each assay (Table 1); and (3) purified phlorotannin fractions from *Sargassum vestitum* and *Ecklonia radiata*. The choice of a standard curve substantially affected the measured estimates of phenolic content for most species. For example, estimated levels of phenolics in the DMBA assay for *Sargassum vestitum* varied between 1.8% dry wt (phloroglucinol reference) and 24.6% dry wt (*S. vestitum* phlorotannin reference), depending on the reference used. Similarly, the Folin-Denis assay resulted in estimates varying from 6.1% dry wt (phloroglucinol reference) to 23.1% dry wt (phlorotannin reference) for *Sargassum vestitum*.

Effects of PVPP

Polyvinylpolypyrrolidone (PVPP) failed to completely remove phlorotannins from all fresh algal extracts except those of *Ecklonia radiata*. Extracts of most algae showed significant color formation (up to 40% of original absorbance) in the DMBA assay after treatment with PVPP (Figure 2).

TABLE 2. PHLOROTANNIN CONTENT (PERCENT DRY WEIGHT) OF 11 SPECIES OF ALGAE USING THREE DIFFERENT STANDARDS FOR FOLIN-DENIS AND DMBA ASSAYS[a]

Algae	Standard 1, PG reference ± SD	Standard 2, overall regression reference ± SD	Standard 3, phlorotannin reference ± SD
Folin-Denis			
Er (L)	5.08 ± 1.78	3.53 ± 1.16	8.9 ± 2.94
Sv (F)	6.10 ± 0.75	10.5 ± 1.3	23.1 ± 2.8
Sl (F)	1.88 ± 0.45	3.41 ± 0.26	not done
Cm (F)	9.90 ± 1.10	16.5 ± 1.8	not done
Pc (F)	2.08 ± 0.64	3.70 ± 1.05	not done
Dm (D)	0.33 ± 0.016	0.58 ± 0.03	not done
Da (D)	5.67 ± 1.20	10.7 ± 1.5	not done
Dd (D)	1.29 ± 0.09	2.15 ± 0.14	not done
Hs (D)	5.00 ± 0.65	8.60 ± 1.16	not done
Zd (D)	6.79 ± 0.75	11.4 ± 1.2	not done
Eb (E)	0.95 ± 0.41	1.93 ± 0.92	not done
DMBA			
Er	1.12 ± 0.45	7.7 ± 3.0	6.01 ± 2.31
Sv	1.80 ± 0.38	11.2 ± 2.0	24.6 ± 2.9
Sl	−0.04 ± 0.10	2.50 ± 0.85	not done
Cm	4.30 ± 0.65	24.3 ± 3.1	not done
Pc	1.97 ± 0.33	5.80 ± 1.50	not done
Dm	0.19 ± 0.13	1.73 ± 0.11	not done
Da	0.04 ± 0.22	3.90 ± 0.92	not done
Dd	−0.06 ± 0.09	1.36 ± 0.04	not done
Hs*	9.45 ± 1.06	40.4 ± 7.5	not done
Zd*	7.58 ± 1.62	50.8 ± 6.1	not done
Eb	0.264 ± 0.28	0.959 ± 0.409	not done

[a] Er = *Ecklonia radiata*, Sv = *Sargassum vestitum*, Sl = *S. linearifolium*, Sg = *S. globulariaefolium*, Cm = *Cystophora moniliformis*, Pc = *Phyllospora comosa*, Dm = *Dilophus marginatus*, Da = *Dictyopteris acrostichoides*, Dd = *Dictyota dichotoma*, Hs = *Homoeostrichus sincalarii*, Zd = *Zonaria diesingiana* Eb = *Endarachne binghamiae*; L = Laminariales, F = Fucales, E = Ectocarpales, D = Dictyotales. Data are means and standard deviations for $N = 6$ samples for all species except *E. radiata* ($N = 9$). Note that negative values reflect the high y intercept for phloroglucinol relative to absorbances for phlorotannins. An asterisk indicates an alga with a high phloroglucinol content, suggesting a phloroglucinol reference is appropriate. Overall regression reference from Table 1.

PVPP also failed to completely remove from solution purified phlorotannins extracted from *Carpophyllum maschalocarpum*, *Fucus vesiculosus*, *E. radiata*, and *S. vestitum* (Figure 2). The difference in the efficacy of PVPP in removing *E. radiata* phlorotannins from fresh extracts versus "pure" fractions is unexplained, although this may be due to qualitative differences in specific polymers present in the two samples.

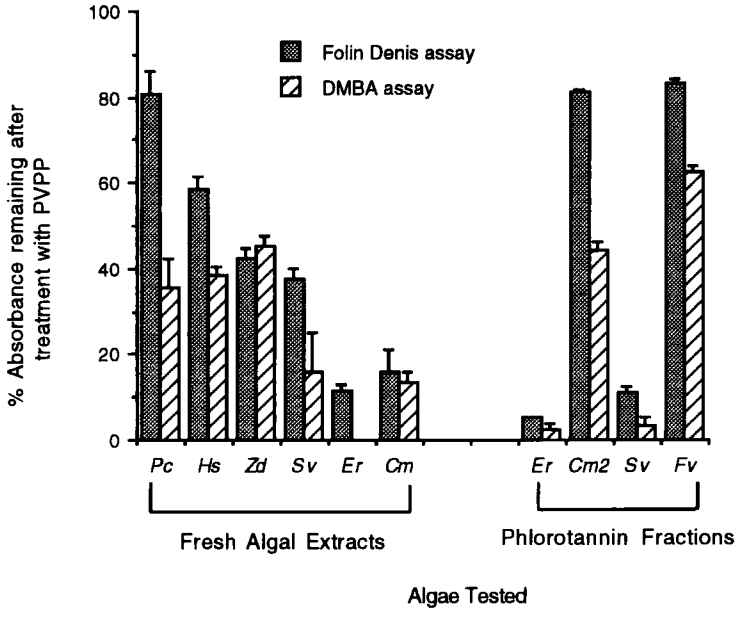

FIG. 2. Percent of initial absorbance remaining after treatment of fresh extracts and purified algal phlorotannins with PVPP. If PVPP is effective at removing phlorotannins, absorbance in the DMBA assay should be zero. (*Pc* = *Phyllospora comosa*, *Hs* = *Homoeostrichus sinclarii*, *Zd* = *Zonaria diesingiana*, *Er* = *Ecklonia radiata*, *Cm* = *Cystophora moniliformis*, Cm_2 = *Carpophyllum maschalocarpum*, *Sv* = *Sargassum vestitum*, *Fv* = *Fucus vesiculosus*).

Sensitivity of Prussian Blue Assay to Interferences

If the Prussian blue assay is less sensitive to interfering compounds than the Folin-Denis assay, then the slope of the line relating estimates of phenolic levels from the two assays should be significantly less than one. For phenolic levels in nine species of New Zealand brown algae, measured in two seasons, the regression had a slope of 0.983 with 95% confidence limits of 0.825 and 1.14 (Figure 3). Thus the Prussian blue assay does not differ significantly from the Folin-Denis assay in sensitivity to interfering compounds present in algal extracts.

Phenolic Levels in Red and Green Algae

Levels of reactive substances (nonphlorotannins) measured by the Folin-Denis and Prussian blue assays for red and green algae from California were mostly $<0.6\%$ by dry wt (Folin-Denis overall mean = 0.50%, Prussian blue

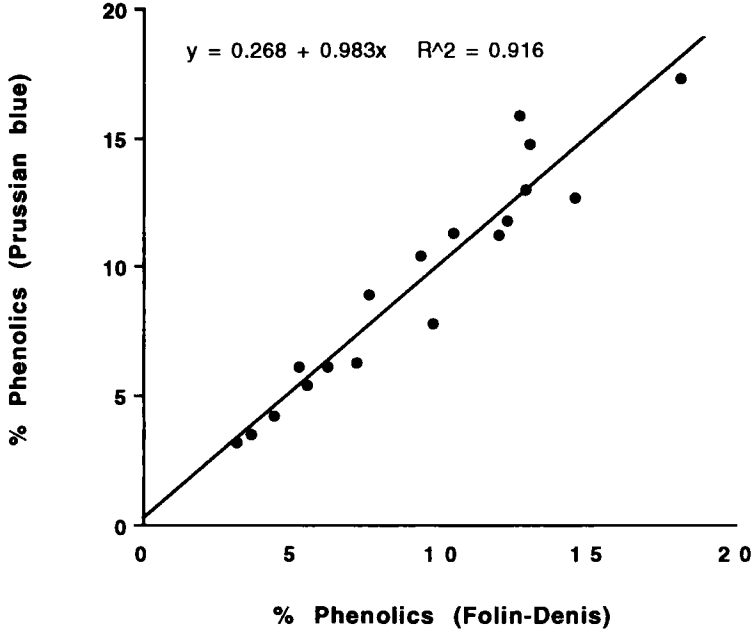

FIG. 3. Phenolic content of nine species of New Zealand algae for summer and autumn measured via the Prussian blue versus the Folin-Denis assay. Regression analysis of mean phenolic levels in each assay ($N = 5$) for each species is highly significant ($P < 0.0001$) with slope = 0.983. The slope is not significantly different from 1 (see text). Species of algae used were *Carpophyllum flexuosum*, *C. maschalocarpum*, *C. angustifolia*, *C. plumosum*, *Ecklonia radiata*, *Sargassum sinclarii*, *Xiphophora chondrophylla*, *Lessonia variegata*, and *Cystophora torulosa*.

overall mean = −0.22%). Higher levels (mean of ≈ 1%) were observed in one species, *Rhodomela larix*; however, this species contains halogenated phenolic monomers at up to 1.2–3.8% dry wt (Phillips and Towers, 1982), which should react in these assays.

Five of the seven New Zealand red and green species assayed also contained relatively low levels of reactive substances. Two species, *Vidalia colensoi* and *Melanthalia abscissa*, had substantially higher levels of reactive substances in both assays (Table 3). The overall average of reactive substances from 17 red and green algal species was 0.50% (Folin-Denis) and 0.09% (Prussian blue). In contrast to the data in Figure 3, this suggests that the Prussian blue assay may be less sensitive to nonphlorotannin interferences than the Folin-Denis assay. However, the large negative values for some species in the Prussian blue

TABLE 3. PERCENT DRY WEIGHT OF PHENOLIC SUBSTANCES IN RED AND GREEN ALGAE FROM CALIFORNIA AND NEW ZEALAND AS MEASURED BY FOLIN-DENIS AND PRUSSIAN BLUE ASSAYS (N = 5 IN ALL CASES)

	Folin-Denis (mean % dry weight ± SD)	Prussian blue (mean % dry weight ± SD)
California		
Red algae		
Endocladia muricata	0.43 ± 0.07	0.05 ± 0.09
Halosaccion glaudiform	0.49 ± 0.3	−1.9 ± 0.82
Iridaea flacida	0.34 ± 0.123	0.25 ± 0.15
Mastocarpus papillata	0.30 ± 0.09	−0.24 ± 0.17
Porphyra sp.	0.38 ± 0.05	0.41 ± 0.25
Prinotus lancelota	0.42 ± 0.34	0.61 ± 0.62
Rhodomela larix	1.07 ± 0.43	0.95 ± 0.23
Green algae		
Chaetomorpha sp.	0.7 ± 0.12	−0.84 ± 0.21
Cladophora sp.	0.52 ± 0.1	−0.56 ± 0.36
Ulva sp.	0.38 ± 0.15	−0.94 ± 0.16
California overall mean	0.50	−0.22
New Zealand		
Red algae		
Vidalia colensoi	1.33 ± 0.26	1.18 ± 0.14
Melanthalia abscissa	1.73 ± 0.23	1.41 ± 0.16
Prilonia sp.	0.67 ± 0.5	0.47 ± 0.47
Gigartina circumcincta	−0.15 ± 0.12	0.21 ± 0.07
Pterocladia lucida	−0.02 ± 0.003	0.19 ± 0.06
Green algae		
Ulva sp.	−0.08 ± 0.15	0.31 ± 0.12
Codium fragile	0.19 ± 0.15	−0.07 ± 0.41
New Zealand overall mean	0.53	0.53

(which contribute to the lower overall mean) are not easily explained and cast some doubt on the utility of this assay for algae. A paired t test across all species from California and New Zealand, using species means as individual replicates, indicates that the Prussian blue values are in fact significantly less (P = 0.0396, df = 16).

As predicted from the chemistry of the DMBA–phlorotannin reaction (Ribéreau-Gayon, 1972), analysis of aqueous methanolic extracts of eight species of red and green algae in the DMBA assay (five of which contain nonpolar metabolites such as terpenoids or acetogenins) yielded essentially no "phenolics" (all samples <0.05% dry wt "phenolics"). Moreover, the λ_{max} of these extracts was often shifted higher than 510 nm (e.g., 580 nm for *Laurenica*

rigida). This suggests that although nonpolar compounds may react in the DMBA assay, color production of such reactions will be slight, and will not interfere with estimation of phlorotannin levels.

DISCUSSION

The new DMBA assay described here has a number of advantages over previously used methods for the analysis of phlorotannins. First, because it is specific to 1,3- and 1,3,5-phenolics, it is insensitive to interferences, measuring only phlorotannins. Second, it is inexpensive and rapid. Third, the DMBA assay was specifically designed to investigate the interaction of phlorotannins with other macromolecules. Thus, this assay can be used to quantitatively measure phlorotannin precipitation by protein (Stern et al., in press), thereby linking together previously disparate methodologies.

The chemical basis for the reaction between aromatic aldehydes such as vanillin (or DMBA), with activated phenolics, such as phloroglucinol, is an electrophilic attack by the aldehyde and must be carried out in a strongly acidic solution (Ribéreau-Gayon, 1972). The product of the reaction is a colored adduct and the intensity of the color is dependent in part on the structure of the aldehyde (Butler et al., 1982). Although vanillin does react with phlorotannins, we took advantage of the increased color yield obtained with DMBA.

The DMBA method can also be easily adapted for determining phlorotannins in plant extracts, gut contents, or other biological samples. Preliminary work in vivo has demonstrated that staining with DMBA can indicate the presence of phlorotannins in the digestive tract of herbivorous marine gastropods and echinoderms (Stern, unpublished data).

One disadvantage of the DMBA assay is that the variation in reactivity of different phlorotannin fractions is somewhat greater than that for the Folin-Denis assay and Prussian blue assay (although fewer phlorotannin fractions were examined for this assay). This variation in reactivity within a given assay presumably reflects differences in the chemical structures of different phlorotannins (Ragan and Glombitza, 1986). Thus the choice of a reference compound becomes particularly important for the DMBA assay, because comparing samples to different standards can result in substantial variation in the estimation of phenolic levels (Table 2).

In an effort to eliminate this problem by finding a commercially available standard, we compared published data on the reactivity of vanillin from a variety of phloroglucinol structural analogs. Information about molar extinction coefficients for these potential reference compounds (e.g., catechin, epicatechin, phloretin, resorcinol, and butein) (Goldstein and Swain, 1963) suggests that they are inappropriate for the DMBA assay.

In some instances, knowledge of the phenolic constituents present in a

particular species can also obviously assist in the appropriate choice of a reference compound. For example, *H. sinclarii* contains substantial amounts of phloroglucinol (determined by thin layer chromatography, unpublished data), as do many species of *Zonaria* (Amico et al., 1981; Blackman et al., 1988; Gerwick and Fenical, 1982) and therefore phloroglucinol would be an appropriate reference for such species. For most algal species neither phloroglucinol nor catechin were suitable as references for DMBA because DMBA responds too strongly to these substances relative to other phlorotannins and therefore significantly underestimates the mass of the phlorotannin measured.

One potential disadvantage of DMBA is that, like vanillin, it can form chromophores with some nonpolar metabolites (under different conditions from this assay and absorbing at different wavelengths than 510 nm) that may interfere with quantitative determinations of phlorotannins in algal extracts (R. de Nys, personal communication). However, all five red and green algae tested with DMBA known to contain nonpolar secondary metabolites yielded negligible values ($<0.05\%$ dry wt) and thus such metabolites are unlikely to pose a problem.

Like DMBA, the Prussian blue and Folin-Denis tests respond differently to different phlorotannins, but with less marked variation. This differential variation is due to differences in chromophore formation. In the Folin-Denis assay oxidation of phenols is coupled with the reduction of phosphomolybdic and phosphotungstic acids to stable chromophores (Ragan and Glombitza, 1986). Thus, although the reaction does not incorporate the phenols into the colored product, it is stoichiometrically dependent on the number of free hydroxyl groups (Ragan and Glombitza, 1986). Aldehydes such as vanillin and DMBA react with the aromatic compound at positions between meta-substituted hydroxyl groups to yield a colored product (Ribéreau-Gayon, 1972).

Ragan and Glombitza (1986) stated that the ratio of color formation between vanillin (and, thus, by implication the vanillin analog DMBA) and Folin-Denis, relative to phloroglucinol, is indicative of phlorotannin molecular weight, with higher ratios indicating lower molecular weights. Our data do not support this for the DMBA assay, as the highest values for this ratio (other than for the monomer phloroglucinol; Table 1) were observed for the low-molecular-weight polymers (MW $\approx 10^4$) from *Ecklonia radiata* (van Altena and Steinberg, 1992) and the high molecular weight polymers from *Fucus vesiculosus*. More generally, the usefulness of obtaining molecular weight information by comparing vanillin- or DMBA-type reactions with Folin-Denis may be limited since: (1) vanillin and DMBA may react preferentially with terminal groups on the molecule, as is the case for terrestrial tannins (Butler et al., 1982); (2) variation in ether and aryl linkages alter reactivity in an unpredictable manner if the structure is unknown; (3) phlorotannins extracted from seaweeds probably span a range of molecular weights; and (4) chromophore production in Folin-Denis is due to

both the number of oxidizable groups as well as their redox potential (which depends on the surrounding molecular environment).

This lack of a consistent relationship between DMBA–FD ratios and molecular weight is unfortunate because molecular weight may explain some of the variation in responses of marine herbivores to phlorotannins. In particular, Boettcher and Targett (1992) showed that variation in the molecular weight of different phlorotannin fractions from *Lobophora variegata* and *Ascophyllum nodosum* significantly affected assimilation efficiencies in the marine herbivorous fish *Xiphister mucosa*, with high-molecular-weight phlorotannin fractions the most inhibitory. In contrast to these results, Steinberg and van Altena (1992; their Figure 7a) and van Altena and Steinberg (1992) found no clear evidence for a relationship between feeding deterrence and phlorotannin molecular weight. Not surprisingly, the importance of molecular weight in the phlorotannin–herbivore interaction is likely to depend on the herbivore and phlorotannins in question and the kind of response examined (e.g., feeding deterrence versus physiological measures, etc.).

The data presented here for the DMBA assay indicate that it is the method of choice for studies of variation in phlorotannin levels in a single species or a group of closely related species. In such a case a purified phlorotannin fraction should be extracted from the algae and used as a standard. This will eliminate difficulties due to differences between the reactivity of the standard and that of the samples.

The appropriate method for broad comparisons among many species from different taxa (Steinberg 1989, Targett et al., 1992) is less clear. The problems associated with using different reference compounds were readily evident for the three colorimetric assays examined in this study. Because a single appropriate reference compound is not readily available for the DMBA assay we recommend that an overall regression consisting of many phlorotannin standard curves may be used as a reference (Tables 1, 2). This seems to minimize the error in estimating phenolic levels across many species and taxa (especially when compared to using phloroglucinol as a standard).

The major advantage of the DMBA assay is that it does not measure nonphenolic interferences, as do the Folin-Denis or Prussian blue assays. Based on our analyses of red and green algae, and assuming that levels of nonphenolic interferences are similar in red, green, and brown algae, levels of interferences are likely to account for 0–1.5% by dry weight (mean $\simeq$ 0.5%) of the phenolic levels measured using these assays. This is somewhat higher than the estimate of nonphenolic materials in *Fucus gardneri* measured by Van Alstyne (1995) using the Folin-Ciocalteau assay, but is still probably an acceptable level of error for assays of phenolic-rich algae, which can have levels of phenolics exceeding 10% by dry weight. Estimates of phenolic levels in more phenolic-poor species may be significantly affected by such levels of interferences, how-

ever. Although levels of reactive nonphenolic substances in *F. gardneri* (Van Alstyne, 1995) were low, the Folin-Ciocalteu assay does not resolve the problem of interferences for brown algae in general. It does have an advantage over the Folin-Denis assay in that it does not suffer from precipitate formation and thus can be used with small sample volumes. The DMBA assay also only uses small volumes of samples and reagents.

Measurement of phenolic levels using the Folin-Denis assay before and after mixing the extract with PVPP does not solve the problem of interferences. Although PVPP has been used in Folin assays to correct estimates of phenolic levels for interferences (e.g., Yates and Peckol, 1993), we found that PVPP failed to remove all phlorotannins from solution, and thus this method can significantly underestimate the levels of phlorotannins in an extract. Van Alstyne (1995) also questioned the utility of PVPP in these assays, although Targett et al. (1995) demonstrated reliable removal of phlorotannins after determining optimal conditions. However, optimal conditions are likely to vary for each phlorotannin–PVPP interaction.

Our data reflect on the accuracy of previously reported algal phenolic levels in one final respect. That is, using phloroglucinol as a reference compound in the Folin-Denis or DMBA assays significantly underestimated the phlorotannin content of most species assayed, relative to using an overall regression standard or purified phlorotannin fractions (Table 1). These differences undoubtedly reflect differences in the chemistry between phloroglucinol and phlorotannins and emphasize the need to continue to improve techniques for specifically quantifying phlorotannins.

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VOLATILE COMPOUNDS FROM CRABAPPLE (*Malus spp.*) CULTIVARS DIFFERING IN SUSCEPTIBILITY TO THE JAPANESE BEETLE (*Popillia japonica* Newman)

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Abstract—The volatile compounds emitted by leaves of four crabapple cultivars susceptible to damage by Japanese beetles and four relatively resistant cultivars were examined. Twelve compounds, mostly terpene hydrocarbons, were identified from intact leaves. The terpenes (*E*)-β-ocimene, caryophyllene, germacrene D and (*E,E*)-α-farnesene occurred in significantly higher levels in susceptible cultivars, whereas resistant cultivars produced greater amounts of (*E*)-4,8-dimethyl 1,3,7-nonatriene and linalool. The relative attractiveness of the cultivars as determined in a pitfall bioassay, however, was not related to their susceptibility to the Japanese beetle as previously determined by defoliation sustained in the field. The attractiveness of individual cultivars was found to be positively correlated with linalool as a percent of the total volatile blend emitted by leaves. This study and previous work suggest that variation in susceptibility of crabapple cultivars to defoliation by Japanese beetles is not due to the attractiveness of the individual cultivars but rather to nonvolatile components of susceptibility and/or resistance. A scenario for host location by the Japanese beetle is presented.

Key Words—*Popillia japonica*, Coleoptera, Scarabaeidae, crabapple, *Malus* spp., semiochemical, linalool, attractant.

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INTRODUCTION

The Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae), is an introduced pest that feeds upon nearly 300 species of plants in a wide variety of unrelated taxa. Among these, many members of the Rosaceae are especially vulnerable to attack, with apple, cherry, and rose being examples of highly favored hosts (Fleming, 1972).

Olfaction plays a major role in host location by the Japanese beetle (Ahmad, 1982). Many volatile compounds have been shown to be attractive, with the most effective known lure being a 3:7:3 combination of geraniol, phenethyl propionate, and eugenol (Ladd and McGovern, 1980). Smith (1924) and Smith and Hadley (1926), citing the early literature, reported that essential oils attractive to the Japanese beetle all contained geraniol in varying amounts. Similarly, Langford et al. (1943) found that essential oils from apple and peach fruit, rose flowers, and sassafras leaves were attractive to the Japanese beetle and that all contained benzaldehyde, citral, eugenol, geraniol, and linalool in various combinations. The volatile compounds produced by Japanese beetle host plants have not been examined, however, using modern isolation procedures and analytical techniques.

In addition to a role in host location, volatile compounds may also play a key part in host acceptance via olfactory and/or gustatory stimulation (Fleming, 1972; Ahmad, 1982). Evidence to support this view includes the observation that removal of a beetle's antennae drastically reduced feeding rates in leaf-disk feeding assays (Ahmad, 1982). Furthermore, it was found that by applying a solution of eugenol in glycerine to *Ginkgo biloba* leaves, a plant not normally susceptible to Japanese beetles, feeding rates were greatly stimulated (Major and Tietz, 1962).

Recently, Spicer et al. (1995) reported marked differences in susceptibility of 42 crabapple cultivars (*Malus* spp.) to the Japanese beetle. Defoliation ranged from nearly 100% to less than 10% for cultivars grown at the same site. Subsequently, a number of physical and chemical variables of crabapple leaves were examined in an attempt to determine the factors involved in Japanese beetle discrimination between different cultivars (Potter and Spicer, unpublished results). Leaves of preferred cultivars generally contained higher levels of nitrogen and sugars and lower amounts of tannins (ANOVA, orthogonal contrasts, $P < 0.001$). Still, a large proportion of the variation in the beetle's feeding patterns could not be explained by these factors.

Because volatile compounds have been assumed to play an important part in host location and acceptance by *P. japonica* (Fleming, 1972; Ahmad, 1982), we examined the volatile compounds emitted by intact leaves of crabapple cultivars that differ in susceptibility to Japanese beetles. We also compared the relative attractiveness of the cultivars in order to determine if olfaction plays a

role in Japanese beetle discrimination between intact foliage of preferred and non-preferred hosts.

METHODS AND MATERIALS

Insects and Plants. Japanese beetles were captured in the field using traps baited with commercial food-type lures (a 3:7:3 ratio of geraniol, phenethyl propionate, and eugenol) (Trécé Inc., Garwood, New Jersey). Six-year-old crabapple trees were maintained in nursery plots at the University of Kentucky's South Farm in Lexington, Kentucky. The four relatively resistant cultivars examined were Baccata Jackii, David, Harvest Gold, and Jewelberry. In a two-year field study of a separate plot with very high beetle populations, these sustained average defoliation levels of 12.2, 31.1, 17.8, and 10.4%, respectively (Spicer et al., 1995). The four susceptible cultivars were Dolgo, Radiant, Red Splendor, and Royalty. These sustained average defoliation levels of 79.4, 100, 97.5, and 92.8%, respectively (Spicer et al., 1995). Plots used in the present experiments had low to moderate beetle populations.

Volatile Compound Collection and Identification. Leaves used for volatile collections were collected from trees from mid-May through early June, prior to the beginning of beetle flight in mid-June. Between 8:00 and 9:00 AM EST, about 10 g of leaves (detached at the base of the petiole) were collected from one tree of each crabapple cultivar. The leaves were placed in a push–pull volatile collection apparatus that had eight chambers for samples and which has been described elsewhere (Loughrin et al., 1995). During the 4-hr collection period, airflow was maintained through the vacuum side of the apparatus at a rate of 250 ml/min, resulting in adsorption of volatile compounds on Super Q absorbent traps (50 mg; Alltech Associates, Deerfield, Illinois).

At the end of the collection period, the traps were removed and compounds were eluted with 180 μl of an 80:20 mixture of hexane–CH_2Cl_2. Cumene (1000 ng) was added as an internal standard and compounds were quantified by 3.0-μl injections onto a gas chromatograph operated with a 60-sec splitless delay and equipped with a 60-m × 0.32-mm Supelcowax 10 column (polyethylene glycol; Supelco Inc., Bellefonte Pennsylvania). GC operating conditions were as follows: oven temperature 60°C for 1 min then programmmed at 2°C/min; injector 220°C; flame ionization detector 240°C; He carrier linear flow velocity 21 cm/sec. Leaf collections and volatile trapping were replicated five times using different trees, and levels of individual compounds were contrasted between susceptible and resistant cultivars by orthogonal comparisons (Sokal and Rohlf, 1981).

Gas chromatography–mass spectroscopy (GC-MS) was performed on a Hewlett-Packard mass ion detector operated in electron impact mode and inter-

faced to a GC equipped with a 30-m × 0.25-mm HP-5 column (5% phenyl, 95% dimethylsiloxane; Hewlett-Packard Co., Palo Alto, California) run as follows: oven temperature 60°C for 1 min and then programmed at 2°C/min to 220°C; injector 220°C; He carrier linear flow velocity 21 cm/sec. Compound identification was performed by computer library searches and retention-time matches of the natural plant products with authentic standards on the Supelcowax 10 column and a 30-m × 0.32-mm AT-5 column (5% phenyl, 95% dimethylsiloxane, Alltech Associates) operated as described for GC-MS analyses. Authentic samples of volatile compounds were obtained from commercial sources except for (*E*)-4,8-dimethyl 1,3,7-nonatriene, which was donated by J. H. Tumlinson (USDA, Gainesville, Florida), and (*E*,*E*)-α-farnesene and germacrene D, which were isolated from the hydrocarbon fraction of ylang-ylang oil (Gaydou et al., 1986).

Pitfall Bioassays. Leaves were collected from the plots during the early stages of beetle flight to minimize the effects of feeding damage. Attraction of Japanese beetles to intact leaves of susceptible and resistant crabapple cultivars was measured in a pitfall bioassay system (Loughrin et al., 1995), which consisted of eight 50-ml Erlenmeyer flasks in plastic trays with lids (32 × 26 cm, 10 cm deep). The trays were filled with dry sand until level with the mouths of the flasks. About 1 g of leaves of a given cultivar was placed in an individual flask and resistant or susceptible cultivar choices were alternated in the trays in a 4 × 2 matrix. Arrangements for all eight cultivars were randomized for each replicate while maintaining a resistant–susceptible cultivar order.

Thirty female beetles were placed lengthwise down the middle of the trays and allowed to choose between the various flasks. Assays were run at room temperature using overhead fluorescent lighting and were allowed to proceed for 3 hr or until 25 beetles had been captured. The assays were replicated 10 times, and the number of beetles responding to each cultivar was analyzed by Friedman's two-way nonparametric analysis of variance (Hollander and Wolfe, 1973).

RESULTS AND DISCUSSION

The identity and yields of volatile compounds obtained from the crabapple cultivars are presented in Table 1. Ten compounds were identified on the basis of mass spectra and retention-time matches with those of authentic samples on the carbowax and AT-5 columns. In addition, (*Z*)-β-farnesene and (*Z*,*E*)-α-farnesene were tentatively identified on the basis of mass spectroscopy, as no authentic samples of these compounds were available to us.

Yields of identified volatiles ranged from about 150 ng g fresh weight for the cultivar Red Splendor to over 1800 ng/g for Royalty. Both of these are

TABLE 1. VOLATILE COMPOUNDS FROM LEAVES OF CRABAPPLE CULTIVARS DIFFERING IN SUSCEPTIBILITY TO JAPANESE BEETLE

	Yield (ng/g fresh weight)[a]							
	Susceptible cultivars				Resistant cultivars			
Compound	Dolgo	Radiant	Red Splendor	Royalty	Baccata Jackii	David	Harvest Gold	Jewelberry
(*E*)-β-Ocimene	17.4 ± 4.5	43.8 ± 10.6	2.2 ± 1.4	321 ± 62.0	6.2 ± 3.3	29.0 ± 9.0	67.7 ± 22.4	62.4 ± 12.1
(*E*)-4,8-Dimethyl 1,3,7-nonatriene	69.8 ± 11.0	111 ± 26.6	24.3 ± 3.4	139 ± 19.7	102 ± 35.4	66.7 ± 27.1	42.3 ± 13.7	721 ± 25.8
(*Z*)-3-Hexenyl acetate	87.7 ± 33.9	54.3 ± 26.9	100 ± 19.8	23.9 ± 4.1	197 ± 99.2	27.9 ± 19.6	42.3 ± 21.6	234 ± 72.3
(*Z*)-3-Hexenol	5.7 ± 3.6	7.6 ± 5.8	3.6 ± 2.9	6.1 ± 2.9	24.0 ± 10.8	5.0 ± 3.6	16.9 ± 7.4	18.9 ± 3.2
Linalool	13.5 ± 2.1	7.3 ± 2.3	2.7 ± 1.6	5.8 ± 3.6	15.9 ± 7.0	8.7 ± 4.8	6.4 ± 4.0	69.2 ± 8.6
Caryophyllene	6.2 ± 2.9	34.8 ± 7.4	[b]	112 ± 26.8		3.1 ± 1.9		23.1 ± 9.1
(*E*)-β-Farnesene	3.4 ± 3.4	6.2 ± 3.9	6.8 ± 3.4	5.7 ± 3.9	6.6 ± 4.0	14.4 ± 10.4		6.8 ± 4.2
(*Z*)-β-Farnesene		130 ± 31.5		242 ± 53.9	6.9 ± 4.4	154 ± 93.1	233 ± 64.9	
Germacrene D		45.7 ± 16.3		184 ± 51.8				6.2 ± 2.8
(*Z*,*E*)-α-Farnesene		8.5 ± 2.5		28.3 ± 9.5		7.6 ± 4.1	9.8 ± 1.8	
(*E*,*E*)-α-Farnesene	12.9 ± 2.3	143 ± 31.5	14.1 ± 6.1	759 ± 235	20.8 ± 7.3	124 ± 7.3	227 ± 52.6	50.4 ± 5.3
Methyl salicylate		10.4 ± 4.4		12.4 ± 6.4		21.1 ± 14.5	25.2 ± 9.4	5.3 ± 3.4
Total Identified	217	603	154	1840	379	462	671	1200

[a] Data represent the mean of five replications ± standard error.
[b] Compound not detected or occurs at less than 2 ng/g fresh weight.

susceptible cultivars. The differences in total yields from the various crabapple cultivars were largely due to variation in the levels of terpene hydrocarbons. For instance, yields of the sesquiterpene (*E*,*E*)-α-farnesene varied from about 13 ng/g for the cultivar Dolgo to over 750 ng/g for Royalty. Similar variations in yield from the various cultivars were noted for the monoterpene (*E*)-β-ocimene and the methylene sesquiterpene (*E*)-4,8-dimethyl 1,3,7-nonatriene.

The relatively high yield of terpene hydrocarbons emitted by crabapple leaves and the marked variation in their yields between the various cultivars within and between resistance groupings is of interest. A number of these terpenes have been shown to be insect attractants. For instance, (*E*,*E*)-α-farnesene is an attractant and oviposition stimulant for the codling moth (*Laspeyresia pomonella*), a serious pest of apples in the Unites States (Sutherland et al., 1977). (*E*,*E*)-α-Farnesene is also a component of the volatile blend emitted by calling male Mediterranean fruit flies (Heath et al., 1994). Caryophyllene has been determined by electroantennogram studies to be attractive to the damson-hop aphid, *Phorodon humuli* (Campbell et al., 1993).

When the yields of volatile compounds from crabapple leaves were grouped according to susceptibility or resistance of the various cultivars to the Japanese beetle (Table 2), a number of significant differences were noted. Specifically, the terpenes caryophyllene, germacrene D, and (*E*,*E*)-α-farnesene occurred in significantly higher levels in susceptible cultivars while (*E*)-4,8-dimethyl 1,3,7-nonatriene and linalool occurred in significantly higher amounts in resistant cultivars ($\alpha = 0.05$, ANOVA, orthogonal contrasts).

To determine if the differences in volatile emission from leaves of the different crabapple cultivars were related to their relative attractiveness to Japanese beetles, we conducted pitfall bioassays in which the beetles were allowed to choose between equal weights of leaves (Table 3). We also wished to determine if beetles discriminate between the various cultivars based on their relative susceptibility to feeding damage. The various cultivars differed significantly in attractiveness to Japanese beetles (Friedman statistic = 15.4; $\alpha = 0.012$, χ^2 approximation). The relative rankings of the cultivars, however, were not related to their degree of susceptibility to damage by Japanese beetles ($r = 0.579$, $P = 0.135$). Of the four most attractive cultivars, three were previously shown to be highly resistant to defoliation in the field (Spicer et al., 1995). Thus our results suggest that susceptibility or resistance of particular crabapple cultivars to damage by Japanese beetles is not necessarily correlated with the relative attractiveness of intact leaves. Rather, variation in resistance may be affected more by differences in nonvolatile biochemical components affecting palatability of leaves (Spicer et al., 1995) or differences in feeding-induced volatiles (Loughrin et al., 1995).

To further explore what compounds or combinations of compounds were

TABLE 2. YIELD OF VOLATILE COMPOUNDS FROM LEAVES OF CRABAPPLE CULTIVARS GROUPED BY SUSCEPTIBILITY AND RESISTANCE TO JAPANESE BEETLE

Compound	Yield (ng compound/g fresh weight)[a]		
	Susceptible cultivars	Resistant cultivars	P^b
(*E*)-β-Ocimene	96.1 ± 33.3	41.3 ± 8.5	<0.001
(*E*)-4,8-Dimethyl 1,3,7-nonatriene	86.2 ± 12.9	234 ± 66.0	<0.001
(*Z*)-3-Hexenyl acetate	66.6 ± 12.9	125 ± 32.5	0.595
(*Z*)-3-Hexenol	5.7 ± 1.4	16.2 ± 3.6	0.243
Linalool	7.3 ± 1.5	25.1 ± 6.6	0.018
Caryophyllene	38.3 ± 12.1	7.0 ± 3.1	<0.001
(*E*)-β-Farnesene	5.6 ± 1.7	6.9 ± 3.0	0.699
(*Z*)-β-Farnesene	93.5 ± 27.5	98.7 ± 34.5	0.851
Germacrene D	57.4 ± 21.3	1.6 ± 0.9	<0.001
(*Z,E*)-α-Farnesene	9.2 ± 3.5	4.3 ± 1.4	0.060
(*E,E*)-α-Farnesene	232 ± 89.4	106 ± 27.3	0.014
Methyl salicylate	6.2 ± 2.2	12.9 ± 4.7	0.441
Total Identified	704 ± 183	679 ± 89.5	0.874

[a]Mean of five determinations ± standard error
[b]ANOVA, orthogonal contrasts

TABLE 3. MEAN NUMBER OF BEETLES CAUGHT IN PITFALL BIOASSAYS AND RANK SUM OBTAINED FROM FRIEDMAN TWO-WAY NONPARAMETRIC ANALYSIS OF VARIANCE

Cultivar	Mean number of beetles caught[a]	Mean rank[b]	Average % defoliation[c]
Dolgo	5.0 ± 1.2	5.70	79.4
Baccata Jackii	4.8 ± 1.1	5.55	12.2
David	4.5 ± 1.3	5.25	31.1
Jewelberry	4.2 ± 0.7	5.60	10.4
Royalty	2.9 ± 1.3	3.80	92.8
Harvest Gold	2.5 ± 1.1	3.60	17.8
Red Splendor	1.7 ± 0.7	3.45	97.5
Radiant	1.4 ± 0.5	3.05	100.0

[a]Mean of 10 determinations ± standard error.
[b]$P = 0.031$ (χ^2 approximation), Friedman statistic = 15.40 with 7 *df*.
[c]Data from Spicer et al. (1995).

most closely correlated with relative attractiveness of the crabapple cultivars to Japanese beetles, we conducted correlation analyses on the cultivar rankings obtained from the Friedman test with absolute amounts of individual compounds from each cultivar and their relative percentage of the total identified volatile blend from each cultivar. There was no relationship between the absolute levels of individual compounds and the relative attractiveness of the cultivars in the pitfall bioassays. However, relative attractiveness was significantly correlated with linalool as a percent of the total identified blend ($r = 0.833$, $P = 0.01$, Table 4). There was also a trend for attractiveness to be correlated with (*E*)-4,8-dimethyl 1,3,7-nonatriene as a percent of the identified blend ($r = 0.652$, $P = 0.08$). Correlation between relative attractiveness and the combined percent of the total blend for both of these compounds was also significant ($r = 0.841$, $P = 0.046$).

Geraniol has been widely reported as a volatile constituent of Japanese beetle host plants (Smith, 1924; Smith and Hadley, 1926; Langford et al., 1943; Fleming, 1972). Linalool, however, readily undergoes acid-catalyzed rearrangement to geraniol (Bauer and Garbe, 1985). It is likely, therefore, that geraniol was an artifact caused by steam distillation.

The Japanese beetle is known to be attracted to a number of common and widely distributed plant aroma constituents such as linalool (Schwartz and Hamilton, 1968). It has been suggested that a generalist herbivore such as *P. japonica* should be attracted to a variety of compounds present in plant aromas and

TABLE 4. PEARSON'S CORRELATION COEFFICIENTS BETWEEN VOLATILE COMPOUND'S AVERAGE PERCENT OF IDENTIFIED BLEND VS. CULTIVAR MEAN RANK OBTAINED FROM FRIEDMAN TWO-WAY NONPARAMETRIC ANALYSIS OF VARIANCE

Compound	Pearson's correlation coefficient	prob > *P*
(*E*)-β-Ocimene	−0.268	0.522
(*E*)-4,8-Dimethyl 1,3,7-nonatriene	0.652	0.080
(*Z*)-3-Hexenyl acetate	0.233	0.578
(*Z*)-3-Hexenol	0.364	0.376
Linalool	0.833	0.010
Caryophyllene	−0.304	0.464
(*E*)-β-Farnesene	0.054	0.899
(*Z*)-β-Farnesene	−0.400	0.326
Germacrene D	−0.528	0.179
(*Z*,*E*)-α-Farnesene	−0.509	0.197
(*E*,*E*)-α-Farnesene	−0.576	0.135
Methyl salicylate	−0.195	0.643

nonspecific blends containing these compounds (Lance, 1983). More specialized herbivores such as the Colorado potato beetle [*Leptinotarsa decemlineata* (Say)], in contrast, would be attracted to a blend of compounds in a more definite ratio (May and Ahmad, 1983; Bernays and Chapman, 1994). It should be noted, however, that specialist herbivores often exhibit attraction to single test compounds of either common or restricted occurrence (Finch and Skinner, 1982; Košťál, 1992). The present study supports the hypothesis for generalist herbivores in that linalool seems to be more important in Japanese beetle attraction than any of the other compounds identified from crabapple leaves. The overall character of the blend, however, may be more important in determining attractiveness to the Japanese beetle than the presence of specific compounds in the blend. Of the compounds we identified from crabapple leaves, only linalool may be described as floral. The Japanese beetle is highly attracted to flowers (Fleming, 1972), and so volatile blends in which floral fragrance compounds comprise relatively high proportions of the total blend should presumably be highly attractive.

Recently, we demonstrated how the volatile compounds induced from crabapple leaves by overnight feeding damage by either Japanese beetles or fall webworms (*Hyphantria cunea* Drury) attracted Japanese beetles (Loughrin et al., 1995). In pitfall bioassays, leaves damaged by overnight feeding were significantly more attractive than undamaged leaves. Artificially damaged leaves or leaves freshly damaged by Japanese beetles, in contrast, were not significantly more attractive than undamaged leaves. The blends of volatile compounds induced by overnight insect feeding damage were distinctly floral in character, which may explain their attractiveness to Japanese beetles.

Results from the present study and that of Loughrin et al. (1995) suggest the following scenario for host location and acceptance by *P. japonica*: Beetles that emerge early in the season respond to odors from a wide variety of plants that differ in characteristics such as the levels of feeding stimulants (e.g., sugars) and the levels of defensive compounds, such as alkaloids and/or tannins. If a beetle locates and samples an acceptable host, feeding is stimulated and this induces volatile release from the damaged leaves. If the host is unacceptable, then the beetle would continue investigating other plant odors until an acceptable host is found. Later-emerging beetles then could exploit the induced volatile blends as a more efficient means of host location. This sequence of events may help to explain the aggregative behavior of Japanese beetles, which has previously been suggested to be the result of a beetle-produced aggregation pheromone (Iwabuchi and Takahashi, 1983).

In summary, we have demonstrated variation in the attractiveness of crabapple cultivars to the Japanese beetle. The attractiveness of individual cultivars was most closely correlated with the relative proportion of linalool in the overall blend of aroma compounds emitted by leaves. While the beetles exhibited

differential attraction to intact leaves of different crabapple cultivars, this was not related to variation in susceptibility to defoliation by Japanese beetles in the field. Volatile compounds could still be important factors in determining the amount of defoliation by Japanese beetles in that the blend of volatile compounds induced by feeding damage (Loughrin et al., 1995) may vary in attractiveness among crabapple cultivars. Future research may resolve whether feeding-induced volatile compounds play a role in discrimination among plant cultivars or plant species.

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VOLATILE COMPONENTS IN DORSAL GLAND SECRETIONS OF THE COLLARED PECCARY, *Tayassu tajacu* (Tayassuidae, Mammalia)

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Abstract—Secretions of the dorsal gland of free-ranging adult male and female collared peccaries (*Tayassu tajacu*) were analyzed by gas chromatography–mass spectrometry. Both sexes contain (2*E*,6*E*,10*E*)-geranylgeraniol; squalene (all *E* isomer); and the following isomers of springene, a diterpene homolog of β-farnesene: (3*E*,6*E*,10*E*)-α-springene, (3*Z*,6*E*,10*E*)-α-springene, and (6*E*,10*E*)-β-springene. A diterpene alcohol and an additional isomer each of squalene and springene also were observed. Straight- and branched-chain esters abound in the secretions of females, but they were not detected in males.

Key Words—Dorsal gland, collared peccary, *Tayassu tajacu*, geranylgeraniol, springene, squalene, straight-chain esters, branched-chain esters.

INTRODUCTION

All peccaries, *Tayassu* spp. (Tayassuidae), possess a large skin gland, called the dorsal gland, that opens at a nipple-like papilla on the midline of the rump (Sowls, 1984). This gland, unique to the Tayassuidae, contains both sebaceous (holocrine) and sudoriferous (apocrine) secretory tissues that empty from common sacs into a single lumen (Werner et al., 1952; Epling, 1956). Peccaries

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typically rub the secretions of the dorsal gland onto conspecifics, branches, and other objects; they also may forcibly eject glandular fluids into the environment as a stream.

A number of functions have been hypothesized for dorsal gland secretions, including territorial scent-marking (Schweinsburg and Sowls, 1972; Sowls, 1984), herd recognition and cohesion (Schweinsburg and Sowls, 1972; Byers, 1985), alarm signaling (Neal, 1959), and predator repulsion (Epling, 1956). The observation that male collared peccaries (*T. tajacu*) nuzzle and lick the dorsal gland of females before copulating has prompted some authors to suggest that the glandular exudates convey reproductive information (Hannon et al., 1991).

Little is known of the composition of dorsal gland secretions. Radioimmunoassays of secretions from female *T. tajacu* suggest the presence of progesterone, the highest concentrations of which were observed in pregnant individuals (Hellgren, 1984). The strong odor often noted for these secretions is consistent with the emission of volatile compounds, but none has previously been characterized.

We report here the results of an analysis by gas chromatography–mass spectrometry (GC-MS) of the volatile components of the dorsal gland secretions of free-ranging adult male and female *T. tajacu*. Our study indicates the dorsal gland as a new vertebrate source of springene, a diterpene homologue of β-farnesene (Burger et al., 1978, 1981), and it reveals sex differences in secretion composition.

METHODS AND MATERIALS

Adult *T. tajacu* were obtained during February 1994 from a hunting station at the Chaparral Wildlife Management Area in the Rio Grande Plains region in Dimmit and LaSalle counties, Texas, U.S.A. Secretions were obtained within 5 hr of sacrifice by exposing the naked area of skin overlying the gland (Figure 1), placing the mouth of a glass vial over the papillary duct opening, and manually compressing the body of the gland. Secretions from six males were pooled in two vials, each containing samples from three individuals. Secretions from eight females were pooled in two vials; one vial contained samples from six individuals, the other contained samples from two individuals. Several milliliters of hexane were added to each vial, whereupon a light brown supernatant appeared. The vials were sealed with polyethylene-lined caps, transported on dry ice, and stored at $-20°C$.

Samples of secretions were diluted with hexane containing dihexyl sulfide as an internal standard for the retention index. Samples (1 μl) of the resulting solutions were analyzed on a Hewlett-Packard 5890 gas chromatograph with a polydimethylsiloxane column (25 m $\times$ 0.32 mm ID, 0.5-μm film thickness)

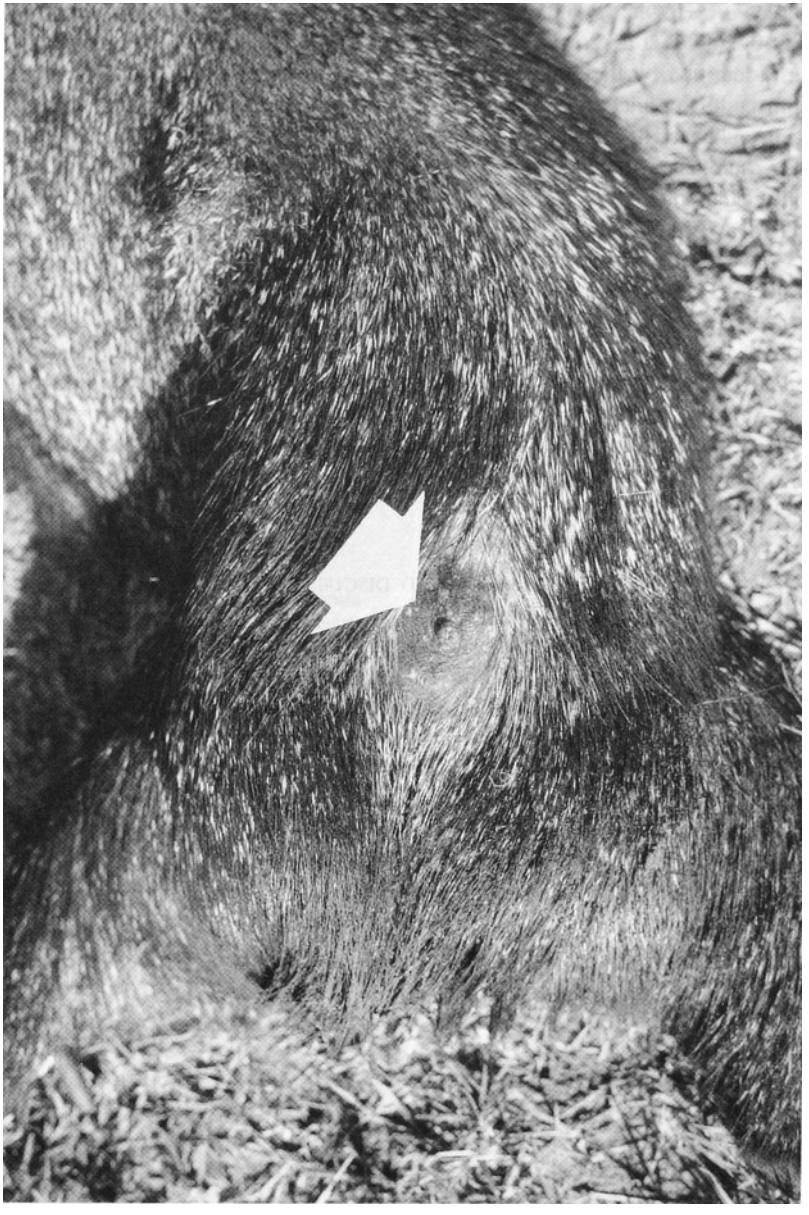

FIG. 1. Posterodorsal view of a *T. tajacu* showing (arrow) naked area of rump with centrally located papillary duct opening of dorsal gland.

coupled to a Hewlett-Packard 5970 mass spectrometer. The temperature program was 40–240°C at 4°C/min, and finally held at 240°C for 20 min. Compounds were identified by their mass spectra. The identities of most compounds were confirmed by coinjection of secretion extracts with authentic compounds, resulting in enhanced GC peaks. Geometric isomerism was defined by GC elution order established for the compounds and their homologs after authentication by ^{13}C nuclear magnetic spectroscopy (Burger et al., 1978; Picket and Griffiths, 1980; Dawson et al., 1982).

Two isomers of springene (α and β) indicated in the secretions were synthesized as described by Burger et al. (1981). Most esters observed were synthesized by refluxing a toluene solution of equimolar proportions of the appropriate carboxylic acid and alcohol in the presence of a strongly acidic ion exchange resin, Amberlyst 15 (Aldrich, Gillingham, U.K.), under automatic water separation until the production of water was complete. Authentic standards of (2*E*,6*E*,10*E*)-geranylgeraniol (Tocris Cookson, Bristol, U.K.) and (6*E*,10*E*,14*E*,18*E*)-squalene (all *E* isomer) (Aldrich, Gillingham, U.K.) were obtained commercially.

RESULTS AND DISCUSSION

Our analysis of the dorsal gland secretions of *T. tajacu* indicates that both males and females contain (2*E*,6*E*,10*E*)-geranylgeraniol and squalene (all *E* isomer) (Table 1); both samples of each sex gave similar results for these and other components. Two compounds, one eluting before squalene (all *E* isomer), the other immediately after, were partially identified. The first of these compounds, characterized as a diterpene alcohol, displayed the following *m/z* (%) values, but no molecular ion: 229 (3), 186 (6), 161 (8), 135 (10), 123 (8), 121 (13), 119 (8), 109 (8), 107 (18), 105 (10), 95 (6), 93 (29), 81 (42), 69 (100), 43 (55), 41 (55). The second compound, characterized as an isomer of squalene, displayed the following *m/z* (%) values: 410 (M^+, 6), 355 (8), 347 (18), 321 (8), 272 (10), 257 (12), 229 (18), 215 (10), 203 (34), 189 (42), 187 (32), 173 (53), 161 (58), 147 (12), 135 (24), 123 (13), 121 (18), 119 (13), 109 (13), 107 (24), 105 (10), 95 (18), 93 (35), 81 (47), 69 (100), 57 (42), 55 (29), 43 (26), 41 (61).

The following isomers of springene, each of which was identified by a molecular ion *m/z* = 272 and a characteristic fragmentation pattern (NIST Standard Reference Data Base, 1990), also were observed in both sexes: (3*E*,6*E*,10*E*)-3,7,11,15-tetramethylhexadeca-1,3,6,10,14-pentaene [(3*E*,6*E*,10*E*)-α-springene]; (3*Z*,6*E*,10*E*)-3,7,11,15-tetramethylhexadeca-1,3,6,10,14-pentaene [(3*Z*,6*E*,10*E*)-α-springene]; and (6*E*,10*E*)-7,11,15-trimethyl-3-methylenehexadeca-1,6,10,14-tetraene [(6*E*,10*E*)-β-springene] (Table

TABLE 1. COMPOUNDS IDENTIFIED BY MASS SPECTRA IN DORSAL GLAND SECRETIONS OF MALE AND FEMALE *T. tajacu*

Peak number[a]	Compound	Male	Female
1	(6*E*,10*E*)-β-Springene[a]	+[c]	+
2	(4*E*,6*E*,10*E*)-3,7,11,15-Tetramethyl-1,4,6,10,14-Pentaene (?)	+	+
3	(3*Z*,6*E*,10*E*)-α-Springene	+	+
4	(3*E*,6*E*,10*E*)-α-Springene[a]	+	+
5	(2*E*,6*E*,10*E*)-Geranylgeraniol[a]	+	+
6	Diterpene alcohol	+	+
7	Squalene (all *E* isomer)[a]	+	+
8	Squalene isomer	+	+
9	Tetradecyl hexanoate[a]	−	+
10	Hexadecyl 4-methylpentanoate[a]	−	+
11	Tetradecyl octanoate[a]	−	+
12	Hexadechyl hexanoate[a]	−	+
13	Pentadecyl 4-methylheptanoate[a]	−	+
14	Pentadecyl octanoate[a]	−	+
15	Hexadecyl 6-methylheptanoate	−	+
16	Hexadecyl octanoate	−	+
17	Heptadecyl 4-methylheptanoate	−	+
18	Hexadecyl 8-methylnonanoate[a]	−	+
19	Hexadecyl decanoate[a]	−	+
20	Hexadecyl dodecanoate[a]	−	+

[a] Peak numbers correspond to those shown in Figure 2.
[b] Components confirmed by coinjection with authentic standards.
[c] +, compound observed; −, compound not observed.

1). The mass spectrum of a fourth springene isomer displayed the following *m/z* (%) values: 272 (M^+, 2), 236 (6), 192 (5), 159 (8), 132 (47), 123 (32), 97 (32), 95 (34), 83 (47), 81 (40), 69 (61), 67 (42), 55 (95), 43 (63), 41 (100). These values are consistent with (4*E*,6*E*,10*E*)-3,7,11,15-tetramethylhexadeca-1,4,6,10,14-pentaene, an isomer not previously reported from nature, although additional spectral data are needed to confirm this structure.

Isomers of springene are known from a variety of natural sources, including a plant (Uegaki et al., 1980), insects (Billen et al., 1988; Fukushima et al., 1990), a crocodilian (Avery et al., 1989), and another mammal, the springbok antelope (*Antidorcas marsupialis*, Bovidae) (Burger et al., 1978, 1981). In the springbok antelope, (3*E*,6*E*,10*E*)-α-springene, (3*Z*,6*E*,10*E*)-α-springene, and β-springene are the volatile products of a skin gland also called the dorsal gland; this organ also is situated on the midline of the dorsum and is hypothesized to release alarm pheromones (Burger et al., 1981). The dorsal gland of the spring-

bok antelope and that of peccaries are not homologous, and we surmise that the abilities of these ungulates to biosynthesize springene isomers evolved independently.

Both pooled samples of secretions from female *T. tajacu* contained high concentrations of the following esters: tetradecyl hexanoate, tetradecyl octanoate, pentadecyl 5-methylheptanoate, pentadecyl octanoate, hexadecyl hexanoate, hexadecyl 4-methylpentanoate, hexadecyl 6-methylheptanoate, hexadecyl octanoate, heptadecyl 4-methylheptanoate, hexadecyl 8-methylnonanoate, hexadecyl decanoate, and hexadecyl dodecanaote (Figure 2). The mass spectra of these compounds display a base peak, $RCO_2H_2^+$, resulting from a double hydrogen transfer. Esters were not detected in either of the two pooled samples of males.

Straight- and branched-chain esters are known from the skin gland secretions of a variety of mammals (see Albone, 1984). In the beaver (*Castor fiber*, Castoridae), esters have been observed in the anal gland secretions of males but not females (Grønneberg and Lie, 1984), the reverse situation from the one

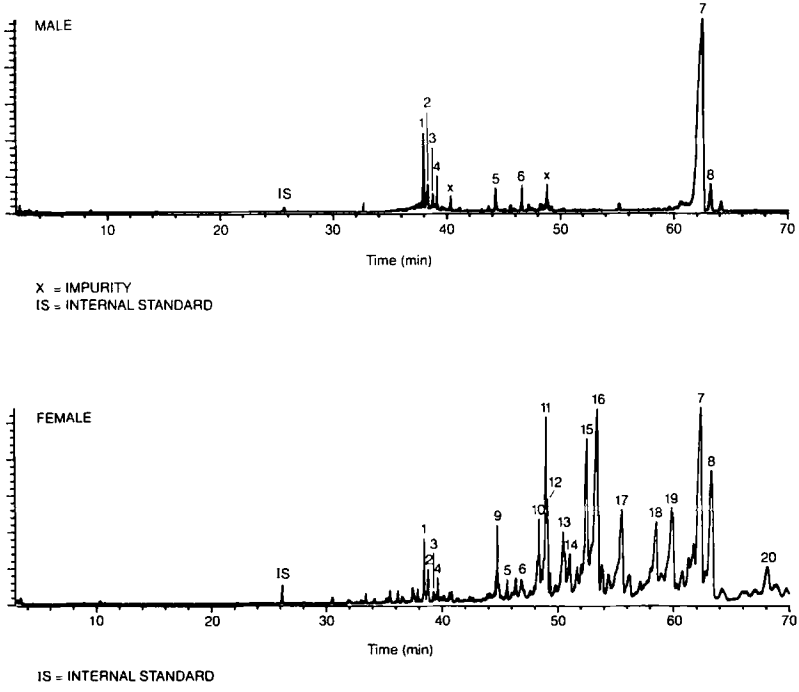

FIG. 2. Chromatograms of dorsal gland secretions of three male and of two female *T. tajacu*. Peak numbers correspond to compounds listed in Table 1.

observed for the secretions of *T. tajacu*. The significance of sex differences in the dorsal gland products of *T. tajacu* is unclear. It is worth noting that the secretory activities of this gland also differ according to sex; sebaceous tissues are better developed in males, while sudoriferous tissues are better developed in females (Hannon et al., 1991). Further investigations are needed to relate these histological observations to the chemistry of the secretions and to the function(s) of the gland.

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STRUCTURE–ACTIVITY RELATIONSHIP OBSERVATIONS FOR THE BAGWORM MOTH PHEROMONE

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Abstract—Structure-activity relationship (SAR) observations were made for the bagworm moth pheromone, (*R*)-2-pentyl decanoate, and a series of analogs with modifications in the alcohol portion of the molecule. Observed attractiveness of these analogs was related to molecular structure and their physical attributes using computational chemistry. Electrostatic potential and Van der Waals (VdW) electrostatic coded surface three-dimensional (3D) maps of the molecular mechanics (MM) minimized lowest energy conformation of the pheromone show that size, shape, charge distribution, and chirality of the molecule are related to attractiveness.

Key Words—SAR, Psychidae, *Thyridopteryx ephemeraeformis*, bagworm moth, sex pheromone, (*R*)-2-pentyl decanoate, Chem-X, molecular modeling.

INTRODUCTION

The bagworm, *Thyridopteryx ephemeraeformis* (Haworth), defoliates ornamentals and trees throughout the Southeast and lower Midwest of the United States (Jones and Parks, 1928). The adult male moth, which flies during the day, is attracted to the adult wormlike female in her pupal case within a silk bag (Neal, 1982) via the female's sex pheromone on her thoracic hairs, which she places at the bottom of the bag until she mates (Leonhardt et al., 1983). After insemination, the female oviposits; the eggs overwinter, and larvae emerge to feed in the spring.

The sex pheromone of the female bagworm moth is (*R*)-2-pentyl decanoate

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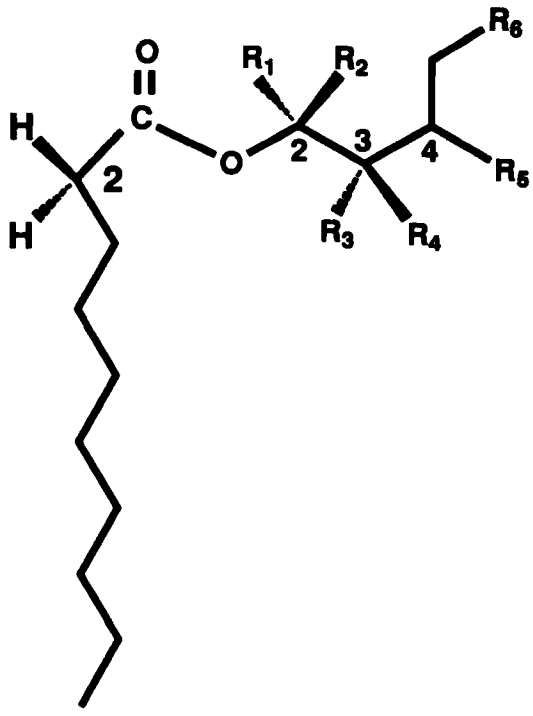

FIG. 1. Bagworm moth pheromone and analogs: (**1a**) $R_1{=}CH_3$, $R_2{=}R_3{=}R_4{=}R_5{=}R_6{=}H$ (pheromone), (**1b**) $R_2{=}CH_3$, $R_1{=}R_3{=}R_4{=}R_5{=}R_6{=}H$ (pheromone antipode), (**2a**) $R_1{=}R_6{=}CH_3$, $R_2{=}R_3{=}R_4{=}R_5{=}H$, (**2b**) $R_2{=}R_6{=}CH_3$, $R_1{=}R_3{=}R_4{=}R_5{=}H$, (**3**) $R_1{=}R_2{=}CH_3$, $R_3{=}R_4{=}R_5{=}R_6{=}H$, (**4a**) $R_1{=}R_3{=}CH_3$, $R_2{=}R_4{=}R_5{=}R_6{=}H$, (**4b**) $R_2{=}R_4{=}CH_3$, $R_1{=}R_3{=}R_5{=}R_6{=}H$, (**4c**) $R_1{=}R_4{=}CH_3$, $R_2{=}R_3{=}R_5{=}R_6{=}H$, (**4d**) $R_2{=}R_3{=}CH_3$, $R_1{=}R_4{=}R_5{=}R_6{=}H$, (**5a**) $R_1{=}R_5{=}CH_3$, $R_2{=}R_3{=}R_4{=}R_6{=}H$, (**5b**) $R_2{=}R_5{=}CH_3$, $R_1{=}R_3{=}R_4{=}R_6{=}H$, (**6**) $R_1{=}R_2{=}R_5{=}CH_3$, $R_3{=}R_4{=}R_6{=}H$, (**7a**) $R_1{=}R_2{=}R_3{=}R_5{=}CH_3$, $R_4{=}R_6{=}H$, and (**7b**) $R_1{=}R_2{=}R_4{=}R_5{=}CH_3$, $R_3{=}R_6{=}H$.

(**1a**) (Figure 1); the antipode (**1b**) is biologically inactive and only acts as a diluent in a racemic mixture (Leonhardt et al., 1983; Klun et al., 1986). The same *R* enantiomeric compound was identified as a sex pheromone in the bagworm, *Oiketicus kirbyi* (Guilding); however, in *O. kirbyi*, the *S* enantiomer inhibited the response of the *R* enantiomer (Rhainds et al., 1994).

To gain information about the structure-activity relationship (SAR) of this chiral pheromone, we report research on the pheromone and a series of analogs based on field studies and computer molecular modeling.

METHODS AND MATERIALS

The bagworm moth pheromone and its analogs (Figure 1) were synthesized via classical esterification procedures (Schwarz and Klun, 1986) with decanoic acid and the following alcohols (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin): (*R*)-2-pentanol, (*S*)-2-pentanol, (±)-2-hexanol, (*R*)-2-hexanol, (*S*)-2-hexanol; 2-methyl-2-pentanol, (±)-3-methyl-2-pentanol, and (±)-4-methyl-2-pentanol. 2,4-Dimethyl-2-pentanol (Wiley Organics, Inc., Coshocton, Ohio) was also used.

(±)-2,3,4-Trimethyl-2-pentanol was synthesized beginning with 3-methylbutanoic acid (isovaleric acid, Aldrich), which was converted to 2,3-dimethylbutanoic acid via butyl lithium in tetrahydrofuran and diisopropylamine to obtain the lithium derivative. The lithium derivative was then treated with methyl iodide and hexamethylphosphoramide (Pfeffer and Silbert, 1970), for an 81.9% yield after distillation at 100°C and 25 torr. The acid chloride formed (Vogel, 1978, p. 498) via thionyl chloride was then converted to the methyl ester with methanol for a 57.8% yield after distillation at 125°C and 760 torr. The desired product was obtained from the methyl ester utilizing methyl magnesium iodide (Vogel, 1978, p. 371).

To determine the purity of the samples, GLC analyses of the analogs (Table 1) were performed on a Shimadzu GC-14A gas chromatograph (GC) equipped with a capillary injector system, a flame ionization detector, and a CR501 data

TABLE 1. PURITY, GLC RETENTION TIME, RETENTION INDEX, AND GC-MS OF RELATED ANALOGS OF FEMALE BAGWORM SEX PHEROMONE

Compound (No.)	Purity (%)[a]	GLC R_t[b]	I_x[c]	e/m[d]
(*R*)-2-Hexyl decanoate (**2a**)	82.6	27.13	1692	M^+-15, 241
(*S*)-2-Hexyl decanoate (**2b**)	92.1	27.15	1693	M^+, 256
(±)-2-Hexyl decanoate (**2a, 2b**)	95.5	27.09	1691	M^+, 256
2-(2-Methyl)pentyl decanoate (**3**)	93.7	25.87	1639	M^+-15, 241
(±)-2-(3-Methyl)pentyl decanoate (**4a–4d**)	46.2	26.70[e]	1674	M^+, 256;
	45.9	26.83[e]	1680	M^+-15, 241
(±)-2-(4-Methyl)pentyl decanoate (**5a, 5b**)	96.9	26.01	1645	M^+, 256
2-(2,4-Dimethyl)pentyl decanoate (**6**)	92.6	27.15	1693	M^+-15, 255
(±)-2-(2,3,4-Trimethyl)pentyl decanoate (**7a, 7b**)	72.2	29.77	1815	M^+, 284

[a] DMS/GLC area that refers to chemical rather than enantiomeric purity.
[b] Dimethylsilicone (DMS), 12 m × 0.2 mm ID fused silica capillary column.
[c] Retention index (Kovats, 1966) on DMS.
[d] DB-1, 60 m × 0.25 mm ID capillary column.
[e] Diastereoisomer.
[f] Diastereoisomers not separable by DB-1.

processor. A Hewlett-Packard 12-m × 0.20-mm-ID dimethylsilicone (DMS) fused silica capillary column was used for the analyses with the following conditions: injector port 200°C; detector 225°C; DMS column, 30°C for 2 min, then programmed at 5°C/min to 180°C and held for 5 min. Total run time was 37 min. A split ratio of 1:50 was used with a helium flow of 1 cm^3/min plus 33 cm^3/min makeup helium. Esters were dissolved in *n*-hexane to make 0.1% solutions, and 1 μl of the solutions was injected for GLC analyses. GC-MS analyses of the analogs (Table 1) were performed on a Finnigan MAT INCOS 50 GC-MS equipped with a Varian 3400 gas chromatograph. The GC component was equipped with a 60-m × 0.25-mm-ID DB-1 capillary column (J&W Scientific) for analyses of the analogs of the female bagworm sex pheromone. The column was held at 100°C for 1 min, then programmed at 20°C/min to 280°C. Analogs were dissolved in *n*-hexane to make 0.01% solutions, and 1 μl of the solutions was injected for GC-MS analyses.

The bagworm female sex pheromone and its analogs were tested for attractiveness for adult males in the field (Table 2). The tests were conducted using Pherocon 1C sticky traps (Trécé, Salinas, California). All traps were baited with rubber stoppers (Thomas Scientific, Catalog no. 1780-J07, Swedesboro, New Jersey) that had been treated with a 60–100 μg/μl methylene chloride solution of compound. In test 1 (Table 2), stoppers were each treated with 1 mg of

TABLE 2. MALE BAGWORM CAPTURES WITH SEX PHEROMONE AND RELATED ANALOGS

Compound (No.)	Total male bagworm captures	
	Test 1, 9/12-10/3/84[a]	Test 2, 10/6-10/21/84[b]
(*R*)-2-Pentyl decanoate[c] (**1a**)	122	245
(*S*)-2-Pentyl decanoate (**1b**)		
(*R*)-2-Hexyl decanoate (**2a**)	43	41
(*S*)-2-Hexyl decanoate (**2b**)	0	0
(±)-2-Hexyl decanoate (**2a, 2b**)	25	22
2-(2-Methyl)pentyl decanoate (**3**)	1	
(±)-2-(3-Methyl)pentyl decanoate (**4a–4d**)	0	
(±)-2-(4-Methyl)pentyl decanoate (**5a, 5b**)	0	
2-(2,4-Dimethyl)pentyl decanoate (**6**)	0	
(±)-2-(2,3,4-Trimethyl)pentyl decanoate (**7a, 7b**)	0	
Blank (empty trap)	1	3

[a] 1 mg/stopper.
[b] 5 mg/stopper.
[c] Female bagworm sex pheromone.

compound; in test 2 (Table 2), stoppers were each treated with 5 mg of compound. Each treated stopper was hung on a paper clip at the center of the trap above its sticky surface. Traps were suspended 1.5 m from the ground and 30 m apart on a chain link fence that surrounded the Beltsville Agricultural Research Center. A randomized complete block design with three replicates and replication over time was used in each test. A blank trap served as the control in both tests. Male captures in the traps were recorded daily or every two days. Freshly treated stoppers were placed in the traps after three to five days in test 1; in test 2, stoppers charged with 5 mg compound were left in the traps throughout the 15 days of the test.

The female bagworm moth pheromone and its analogs were constructed via the computer molecular modeling program, Chem-X (January 1993 VAX version, Chemical Design Ltd, Oxford, England). Lowest-energy conformations were determined using molecular mechanics (Burkert and Allinger, 1982; Clark, 1985) with modified force-field parameters (including Gasteiger charges). Electrostatic potential (Figure 2) and Van der Waals (VdW) electrostatic coded surface three-dimensional (3D) (Figure 3) maps of the bagworm moth pheromone (**1a**) were also constructed.

RESULTS AND DISCUSSION

Previous field tests showed that the *S* enantiomer (**1b**) of the levorotatory bagworm moth pheromone, (*R*)-2-pentyl decanoate (**1a**) (Figure 1, Table 1), is inactive and does not inhibit the attractiveness of the *R* enantiomer (Leonhardt et al., 1983; Klun et al., 1986).

To study the SAR of the pheromone (**1a**), modifications were made in the alcohol portion of the molecule. The alcohol chain was extended by one carbon via synthesis of (±)-1-hexyl decanoate (**2a, 2b**). This racemate was ~20% as active (test 1, Table 2) as the pheromone; the levorotatory *R* (**2a**) enantiomer of this analog was ~35% as active as the pheromone, and the *S* (**2b**) enantiomer was inactive. To confirm the latter findings, test 2 was conducted; the levorotatory *R* enantiomer (**2a**) was 17% as active as the pheromone, and the *S* (**2b**) enantiomer was inactive. In both tests, the levorotatory *R* enatiomer (**2a**) was about twice as active as the racemate (**2a, 2b**). This indicated that the inactive *S* enantiomer was not inhibitory to the *R* enantiomer (**2a**).

Other structural changes to the pheromone molecule were also considered, which contributed to the SAR study. A carbon was added to the pheromone at the 2-pentyl position to give 2-(2-methyl)pentyl decanoate (**3**), which was essentially inactive in field traps. A carbon was added to the pheromone at the 3-pentyl position to give (±)-2-(3-methyl)pentyl decanoate (**4a–4d**), which was inactive. Similarly, a carbon was added to the pheromone at the 4-pentyl position

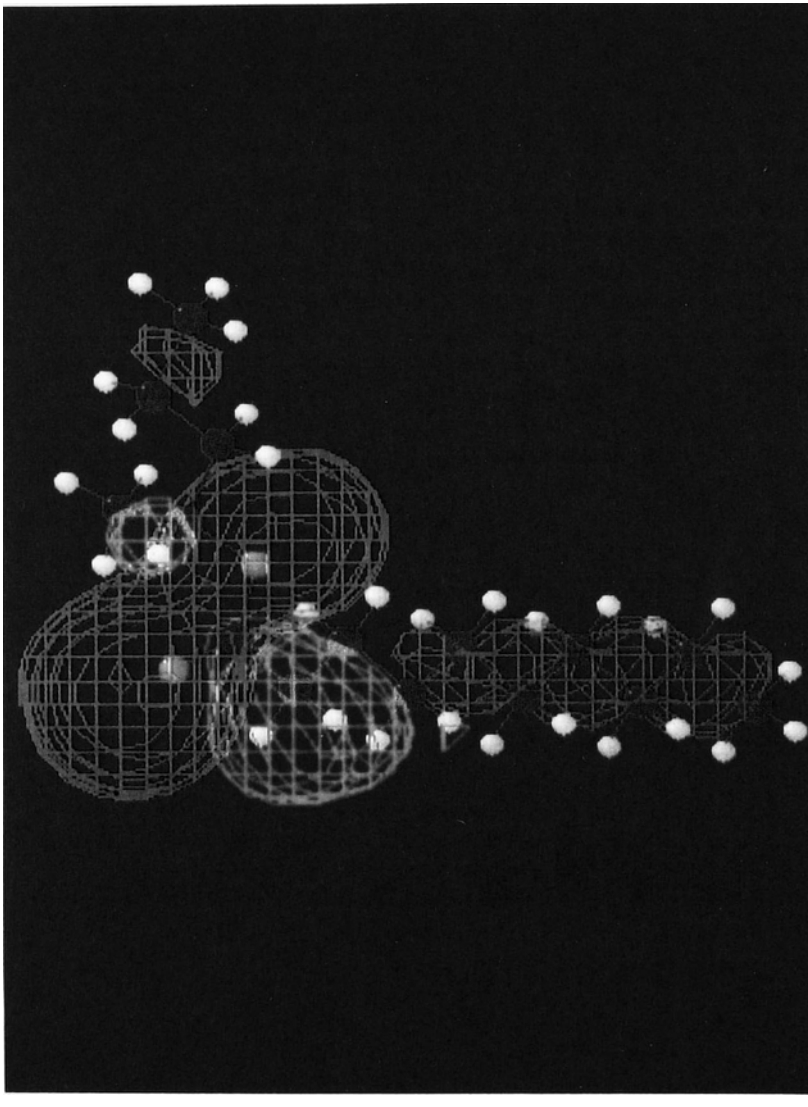

FIG. 2. Electrostatic potential 3D map of bagworm moth pheromone (**1a**). Standard contours: cyano, < -10.0 kcal; red, > 10.0 kcal; significance = 20; grid density = 2.5 contours/Å.

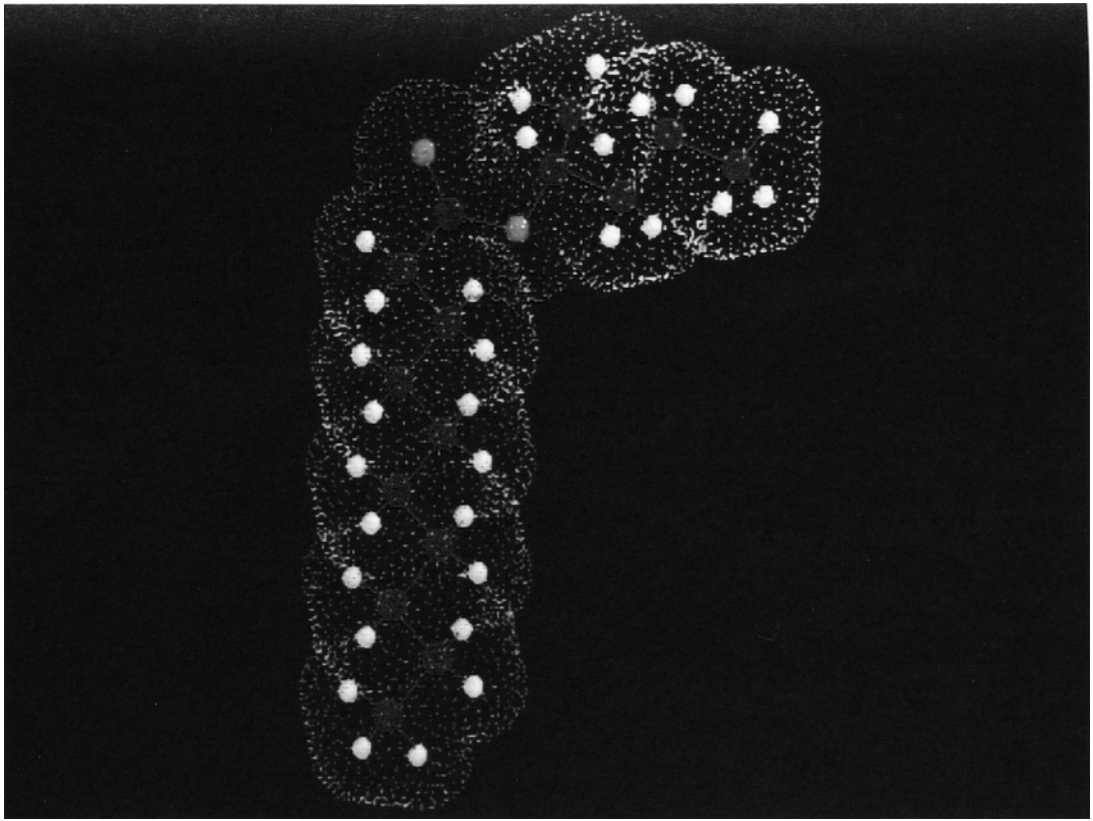

FIG. 3. VdW electrostatic coded surface 3D map of bagworm moth pheromone (**1a**). Standard contours: cyano, < -20.0 kcal; green, < -10.0 kcal; white, < 10.0 kcal; yellow, < 20.0 kcal; red, > 20.0 kcal; significance = 0.95; grid density = 2.5 contours/Å.

to give (±)-2-(4-methyl)pentyl decanoate (**5a, 5b**), which was also inactive; two carbons were added to the basic pheromone structure to give 2-(2,4-dimethy)pentyl decanoate (**6**), which was inactive. Finally, three carbons were added to the basic pheromone structure to give (±)-2-(2,3,4-trimethyl)pentyl decanoate (**7a, 7b**) which was also inactive.

The GLC purity (Table 1) of the analogs was 92+% except for (*R*)-2-hexyl decanoate (**2a**) and (±)-2-(2,3,4-trimethyl)pentyl decanoate (**7a, 7b**), which were 82.6 and 72.2%, respectively. The two diastereoisomers of (±)-2-(3-methyl)pentyl decanoate (**4a–d**) were resolved on the 12-m × 0.20-mm-ID column, but not on the 60-m × 0.25-mm-ID column with the conditions used. All analogs had a parent ion in the GC-MS, except for **2a, 3,** and **6,** which had $M^+ - 15$ ions.

The electrostatic potential map (Figure 2) of the pheromone (MM energy = −1.2071 kcal/mol), (*R*)-2-pentyl decanoate (**1a**), shows partially charged positive areas on the hydrogen at the (*R*)-2 position and on the hydrogens at the α-position of the decanoate chain. There is also a partially charged negative area around the two O atoms because of the electronegativity of these atoms. This could indicate that not only is the chiral steric effect of the (*R*)-2-CH_3 necessary for attractancy, but so is the partially charged positive (*R*)-2-H in relation to the partially charged negative oxygens with adjacent partially charged positive α-hydrogens on the decanoate chain. The volumes of the cyano (< −10 kcal electrostatic potential) and red contours (> 10 kcal electrostatic potential) are 67.8989 $Å^3$ and 17.9873 $Å^3$, respectively; the surface areas of the cyano and red contours are 97.4782 $Å^2$ and 30.7097 $Å^2$, respectively.

The VdW electrostatic coded surface 3D map (Figure 3) of the pheromone gives an indication of the size (volume = 216.47 $Å^3$), surface shape (area = 230.312 $Å^2$), and electrostatic charge distribution that is required for biological activity.

The antipode (**1b**) of the pheromone and **2b** are inactive because they appear right-handed instead of left-handed, with electrostatic potentials on the (*S*)-2-H and the α-hydrogens of the decanoate that are reversed in relation to the potential on the oxygens. In **1a** and **1b,** the energy minimized structure is L-shaped (Figure 3). Chirality determines whether the front or back surface of the L shape contains the electropositive 1-H proton. The large difference in their activity suggests only one surface (i.e., front) is active. Compound **2a** has the correct chirality for activity, but is tempered in activity by steric effects corresponding to extra molecular volume and surface area of a terminal methyl. Analogs that are achiral at position-2 (**3, 6,** and **7a, 7b**) lack the (*R*)-2-H to give the appropriate electrostatic potential distribution. The remaining compounds (**4a–d** and **5a, 5b**) have an (*R*)-2-H in the racemate, but the added substituents cause an increase in the molecular volume and surface area that could exceed the requirements. The volatilities of compounds (Tables 1 and 2)

would be expected to be quite similar except for **7a** and **7b**, with a retention time of 2.6–3.9 min greater than the other compounds.

No modifications were attempted on the acid portion of the pheromone for this study.

CONCLUSIONS

Modifications in the alcohol portion of the levorotatory bagworm moth pheromone, (*R*)-2-pentyl decanoate (**1a**), revealed that the chirality at the 2-pentyl position is absolutely essential for attractiveness since the *S* antipode (**1b**) is not attractive. It was also determined that the most stable conformation is L-shaped. Lengthening the alcohol with a terminal carbon reduced attractiveness by 65–83% as long as the correct chirality (**2a**) was maintained. Steric effects from the addition of a carbon at the 2 (**3**), 3 (**4a–d**), or 4 positions (**5a, 5b**); two carbons at the 2 and 4 positions (**6**); or three carbons at the 2, 3, and 4 positions (**7a, 7b**) of the pheromone resulted in no attractiveness. The bagworm moth allows very few changes in the alcohol portion of the pheromone if attractancy is to be maintained. The dumbbell shape of the electrostatic partial negative charge of the oxygen atoms in relation to the electrostatic partial positive charge of the chiral (*R*)-2-H and the electrostatic partial positive charge of the α-hydrogens on the decanoate chain point toward the importance of the front surface of the L-shaped (**1a**) enantiomer. Size and shape linked to this chiral surface contribute to its attractiveness for bagworm moths.

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TOXICITY OF LIGNANS TO SYMBIOTIC FUNGUS OF LEAF-CUTTING ANTS

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Abstract—Lignans from *Virola sebifera* Aubl., *Virola* sp., and *Otoba parvifolia* (Mkfg.) A. Gentry (Myristicaceae) inhibited the in vitro growth of the fungus cultivated by leaf-cutting ants of the species *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae). A comparison of activity among the lignans was obtained.

Key Words—Lignans, leaf-cutting ants, antifungal compounds, *Virola sebifera*, *Otoba parvifolia*, *Atta sexdens rubropilosa*.

INTRODUCTION

Leaf-cutting ants belonging to the genera *Atta* and *Acromyrmex* are widely distributed in the New World and have been considered to be among the most destructive herbivores in this area (Weber, 1972). In natural ecosystems, ants show a preference for some plant species that are constantly defoliated, while others are not attacked even though abundant and close to their nests (Cherrett, 1968; Hubbel and Wiemer, 1983; Littledyke and Cherrett, 1975; Rockwood, 1976).

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Attraction or repellency of different plants for ants may be influenced by physical and chemical properties of leaves, such as thorns, hardness of the epidermis, hairs, moisture level, nutritional quality, and secondary metabolites (Hubbell and Wiemer, 1983; Hubbell et al., 1984). Chemical defense of plants is generally associated with toxicity to herbivores of secondary metabolites such as alkaloids and terpenoids. (Harborne, 1994). There is some experimental evidence that secondary metabolites may be harmful to leaf-cutting ants and their symbiotic fungus (Howard et al., 1988). In Brazil the native species *Virola sebifera* Aubl. (Myristicaceae) seems not to be cut by leaf-cutting ants in the natural environment. In a preference assay, Bueno et al. (1989) showed that their leaves were not chosen, thus confirming that this plant is not used as a forage source by these ants.

Fernandes et al. (1988) showed that leaves of *V. sebifera* have lignans of both the furofuran and dibenzylbutyrolactone groups. Lignans largely occur in plants, and more than 500 compounds of this class are already known. Lignans are known for their antitumoral, antimitotic, and antiviral activities (MacRae and Towers, 1984).

We have recently (Pagnocca et al., 1990) reported the activity of sesame (*Sesamum indicum*) extracts on the growth of the symbiotic fungus cultivated by leaf-cutting ants of the species *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae). During our continuing work on the isolation of bioactive natural products we have found that lignans can also inhibit the growth of the symbiotic fungus of the leaf-cutting ants *A. sexdens rubropilosa*. Lignans are known to be present in sesame oil. The possibility that they might also be found in the leaves of *S. indicum* prompted us to check some lignans isolated from other plants for activity against the symbiotic fungus of *A. sexdens rubropilosa*.

METHODS AND MATERIALS

Plant Extracts and Lignans Source. Extracts were prepared from leaves of *V. sebifera* Aubl. (Figure 1: **1, 2, 5, 6**), *Virola* sp. (**3, 7, 8**), and fruits of *O. parvifolia* (Mkfg.) A. Gentry (**4**) (Myristicaceae). Fernandes et al. (1988) and Ferreira et al. (1989) isolated some of the lignans used in the experiments, and others (lignans **3, 7,** and **8** from *Virola* sp.) were isolated by H. F. Paulino Fo. (unpublished data).

Microorganism. The fungus was isolated from a laboratory nest of the leaf-cutting ant *A. sexdens rubropilosa* Forel by streaking the spongelike mycelial material (fungal garden) in Petri dishes containing Sabouraud agar supplemented with 200 mg/liter of chloramphenicol and the pH adjusted to 4.0 with 1 N HCl. The nest was maintained in the laboratory with green leaves, mostly of *Eucalyptus alba*. The fungus showed typical characteristics of the ant fungus *Leucocoprinus gongylophorus*, syn. *Rozites gongylophora* Möller (Heim, 1957).

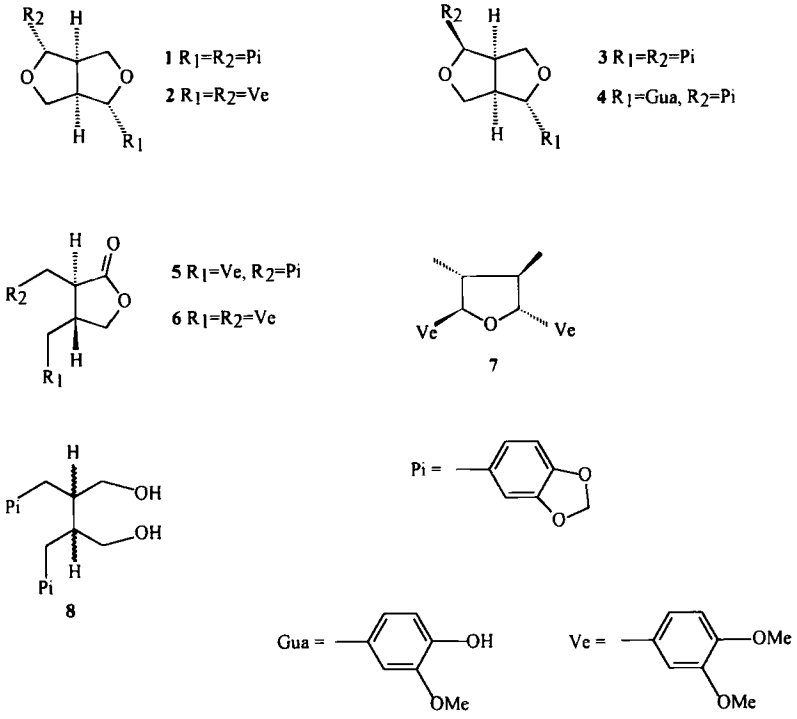

FIG. 1. Structures of the lignans tested. Substitutions in the basic lignan structure include the piperonyl rings (Pi), the guaiacyl group (Gua), and the veratryl group (Ve).

Culture Medium, Inoculum, and Growth Evaluation. The following culture medium (grams per liter) was used for both the assays and maintenance of the culture: glucose, 10.0; sodium chloride, 5.0; Bacto-peptone, 5.0; malt extract, 10.0; and agar, 15.0. The medium (9 ml) was distributed in test tubes (180 × 18 mm) and 1.0 ml of each lignan (10 × concentrate) was added in order to get a final concentration as follows: **1** = 70 μg/ml; **2** = 160 μg/ml; **3, 4, 5, 7, 8** = 200 μg/ml; and **6** = 210 μg/ml. Solutions of concentrated lignans were prepared by adding about 20.0 ml of solvent (dichloromethane) to crystals of pure lignans, and the concentration (10×) reached was a consequence of their yield after the purification procedure. Before addition to the culture medium, the volume was adjusted to give a final concentration near 200 μg/ml except for lignan **1,** which was shown to be active in a lower concentration (Pagnocca et al., 1987). To the controls (10 test tubes) was added 1.0 ml of dichloromethane. The complete medium was autoclaved for 10 min at 120°C. To check if a possible remaining amount of solvent could inhibit fungal development, a

second control was used with water instead of dichloromethane. The mycelium present in six stock culture tubes about 30 days old was transferred aseptically to an all-glass tissue grinder (250 × 25 mm) containing a small amount of peptone solution (0.1 w/v) and gently fragmented. Then, it was diluted with about 100 ml of sterile saline, and 1.0 ml (corresponding to 5.3 ± 1.0 mg of dry weight) was added to tubes containing slanted medium. The tubes were shaken to provide a good distribution of mycelial fragments on the agar slant and incubated in an oven at 25 ± 1°C for 30–35 days. The assays were run with 10 test tubes and repeated twice. After the period of incubation, the fungal growth was estimated macroscopically on the basis of mycelial surface and density and the relative growth was registered and expressed in percentage.

RESULTS AND DISCUSSION

Our previous experience showed that during autoclaving the organic solvent used was evaporated and did not inhibit the fungal development. The same was observed in the current assay.

Lignans varied in their inhibitory effect on the development of the symbiotic fungus from *A. sexdens rubropilosa*. Lignans **3** and **8** did not inhibit fungal growth at 200 μg/ml (Table 1). Lignan **4** (philygenol) from *O. parvifolia* and lignan **5** from *V. sebifera* both inhibited fungal development only slightly at a concentration of 200 μg/ml. Fungal growth was inhibited about 40% by lignan **2** (eudesmin) at a concentration of 160 μg/ml and 60% by lignan **6** from *V. sebifera* at 210 μg/ml. Complete inhibition of fungal development was obtained from lignan **7** (epigalgravin) and **1** (sesamin) at concentrations of 200 μg/ml and 70 μg/ml, respectively (Table 1).

Sesamin is found in different plants (Budowski, 1964) and has been shown

Table 1. INHIBITORY EFFECT OF LIGNANS ON FUNGUS GROWTH

Lignan	Final concentration (μg/ml)	Maximum fungus growth after (30–35 days) (%)
Sesamin (**1**)	70	<20
Eudesmin (**2**)	160	60
Episesamin (**3**)	200	100
Philygenol (**4**)	200	80
Lignan (**5**)	200	80
Lignan (**6**)	210	40
Epigalgavrin (**7**)	200	<20
Lignan (**8**)	200	100

to be a more potent inhibitor of fungal growth than other lignans. Lignan **6** (dibenzylbutyrolactone) also showed good inhibition activity and is an abundant constituent of *V. sebifera* (Fernandes et al., 1988). Furthermore, lignans **6** and **7** showed activities in tubulin binding and antitumor assays together with podophyllotoxin (Kelleher, 1978). Lignans **7** (tetrahydrofuran) and **6** (dibenzylbutyrolactone), and even lignan **2** (furofuran), in spite of inhibiting the fungal growth at a lower concentration than the first two (**1, 7**), had antifungal activity independent of chemical structural type.

Sesamin (**1,** furofuran) has two piperonyl rings (*cis*) linked to the furofuran central ring, while episesamin (**3**) presents the piperonyl rings *trans*. This difference in the stereochemistry maybe responsible for the lack of antifungal activity of episesamin. Eudesmin (**2,** furofuran) presents two veratryl rings (*cis*), and the presence of this group instead of piperonyl, as in sesamin, may lower antifungal activity. Lignan **4** (philygenol) shows one guaiacyl and one piperonyl ring and had weak activity in spite of the rings being *trans*, as in episesamin. This suggests that the guaiacyl group may have more influence than the piperonyl group on antifungal activity. This hypothesis will be tested in future studies when a lignan with two guaiacyl rings will be compared with sesamin. When the dibenzylbutyrolactone lignans **5** and **6** were compared, it was observed that the replacement of one veratryl group by a piperonyl group reduced antifungal activity by about 50%.

Of all components tested, sesamin was the most potent in the inhibition of fungus growth. The presence of lignans in the *V. sebifera* could be the main reason why leaf-cutting ants do not cut their leaves.

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DICHLOROMETHANE ATTRACTS DIABROTICITE LARVAE IN A LABORATORY BEHAVIORAL BIOASSAY

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Abstract—A two-choice laboratory behavioral bioassay was used to demonstrate that dichloromethane elicits the dose-dependent attraction of second-instar western and southern corn rootworms. Preliminary data suggest that second-instar banded cucumber beetles are also attracted to dichloromethane. An eluotropic series of 10 materials, including distilled water, ethanol, methanol, acetone, ethyl dichloroacetate, dichloromethane, diethyl ether, benzene, hexadecane, and hexane, was tested for attraction of western corn rootworm larvae. Dichloromethane was the only one attractive at all doses tested, and orthogonal comparisons revealed a quadratic trend (convex) for responses of larvae to increasing dose. Benzene and hexadecane also attracted larvae, but significantly fewer than dichloromethane, and only at three doses and one dose, respectively. Orthogonal comparisons revealed no linear or quadratic trend for responses of larvae to increasing doses of either compound. Dichloromethane is the first organic compound demonstrated to attract western corn rootworm larvae in the absence of carbon dioxide. Carbon dioxide has previously been reported to attract western corn rootworm larvae either independently or when combined with other organic compounds, and the sensitivity of our bioassay was tested by demonstrating the dose-dependent attraction of western corn rootworm larvae to carbonated water as a carbon dioxide source. We have also demonstrated the attraction of southern corn rootworm larvae to carbon dioxide and propose that carbon dioxide and dichloromethane behave analogously when they interact with chemoreceptor sites on larvae.

Key Words—*Diabrotica virgifera virgifera*, *Diabrotica undecimpunctata howardi*, *Diabrotica balteata*, Coleoptera, Chrysomelidae, semiochemical, structure-activity, host-finding, carbon dioxide, corn, soybeans.

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INTRODUCTION

Several *Diabrotica* species with varying host ranges are associated with corn, cucurbits, or soybeans. The western corn rootworm (WCR), *Diabrotica virgifera virgifera*, (Coleoptera: Chrysomelidae) is the most economically important insect in the United States; requiring more than one billion dollars annually for the reduction of damage caused when its larvae feed on the roots of corn plants (Metcalf and Metcalf, 1993). WCR larvae can survive on the roots of other grasses, completing their immature development on at least 14 species of graminaceous plants and producing fertile adults on at least five species (Branson and Ortman, 1967, 1970). Branson and Ortman (1970) concluded that the larvae are probably oligophagous and that their host range is limited to certain species of the Poaceae, but that corn is still the most favorable host for larval development. The southern corn rootworm (SCR), *Diabrotica undecimpunctata howardi* is also considered a pest of corn, but has a broader host range and has been collected from more than 200 common weeds, grasses, and cultivated crops, including soybeans (Metcalf and Metcalf, 1993). Like the SCR, the banded cucumber beetle (BCB), *Diabrotica balteata* (Coleoptera: Chrysomelidae), also has a broad range and has traditionally been considered a defoliator of cucurbits, but in the past several decades it has developed into an important pest of soybeans in the gulf states (Metcalf and Metcalf, 1993).

Several compounds associated with corn facilitate host-finding by WCR larvae, but none have been reported for SCR or BCB larvae. Carbon dioxide is released from corn roots (Harris and van Bavel, 1957; Massimino et al., 1980) and has previously been demonstrated to attract first-instar WCR (Strnad et al., 1986). Extracts of germinating corn seedlings have been demonstrated to augment attraction of either first- or second-instar WCR to a source of carbon dioxide in laboratory bioassays (Strnad et al., 1986; Strnad and Bergman, 1987; Hibbard and Bjostad, 1988, 1990). Some organic compounds in these extracts have been identified and demonstrated to influence larval behavior in laboratory bioassays with carbon dioxide. Bjostad and Hibbard (1992) reported the combination of 6-methoxy-2-benzoxazolinone (MBOA) and carbon dioxide as a cue for larval host recognition in laboratory bioassays. Field experiments have also supported the role of MBOA as a host recognition cue for larvae (Hibbard et al., 1995). Hibbard et al. (1994) reported that each of three fatty acids that were isolated from corn, including stearic acid, oleic acid, and linoleic acid, when combined with carbon dioxide, served as host recognition cues for larvae in laboratory bioassays.

While developing new bioassay approaches for the isolation of more behaviorally active compounds from corn, we observed the attraction of second-instar larvae to dichloromethane. We then initiated the systematic evaluation of WCR behavioral responses to 10 different materials that comprise a wide range of

polarities. We evaluated the sensitivity of our bioassay by testing the responses of WCR larvae to carbonated water as a carbon dioxide source. The dose-dependent attraction of SCR larvae to carbon dioxide and to dichloromethane was also considered. Attraction of BCB larvae to dichloromethane was tested as well.

METHODS AND MATERIALS

WCR, SCR, and BCB Larvae. Second-instars of WCR and SCR, between 5 and 7 days old and 4–7 mm long, were used for all our experiments. WCR larvae were obtained from the colony in our laboratory that was established and maintained using methods described by Jackson (1986) with modifications by Hibbard and Bjostad (1988). The original source of our WCR was a nondiapausing strain obtained in June 1986 from the USDA-ARS laboratory in Brookings, South Dakota. The colony has been supplemented frequently since then with eggs from French Ag. Research Inc. (R.R. 2, Box 294, Lamberton, Minnesota 56152). SCR larvae (a nondiapausing strain reared on germinating corn seeds) were obtained from French Ag. Research Inc., and bioassays with them began on the day of their receipt. BCB larvae (reared on germinating wheat seeds) between 3 and 5 days old and 3–6 mm long were obtained from Michael Jackson (USDA-ARS, U.S. Veg. Lab., 2875 Savannah Hwy., Charleston, South Carolina 29414) and were used on the day of their receipt.

Bioassay Apparatus. Each bioassay apparatus contained an arena, a stand for the arena, and dispensers for the volatile compounds (Figure 1). Arenas of the two-choice bioassay were constructed from a single 90-mm × 15-mm disposable, polystyrene Petri dish (Becton Dickinson and Company, 2 Bridgewater Lane, Lincoln Park, New Jersey 07035). Two round holes (11 mm diameter) were made in the bottom of the dish, 65 mm opposite from one another, using a brass tube attached to a soldering iron. Filter papers (qualitative, number 4, 90 mm diameter, Whatman International Ltd., Maidstone, England) were cut to a diameter of 86 mm. Two round holes (11 mm diameter) were cut opposite one another in the filter papers with a gasket punch (Snap On Tools Corp., Kenosha, Wisconsin 53140). The filter paper was used to cover the bottom of the Petri dish, with the holes aligned. Stands for the arenas were constructed from 10.5-cm × 15.7-cm pieces of heavy paper (110 lb, Dixon Paper Co., 3900 Lima St., Denver, Colorado 80239). When finished, the two arms of each stand were 45 mm high. Two 4-ml glass shell vials (Baxter Healthcare Corp., Scientific Products Div., McGaw Park, Illinois 60085-6787) were uncapped and placed on the stand, and their open tops were flush with the top of each arm on the stand. An arena was set on the stand such that the two holes in the bottom of it were aligned with the openings in the tops of the two shell vials. Larvae

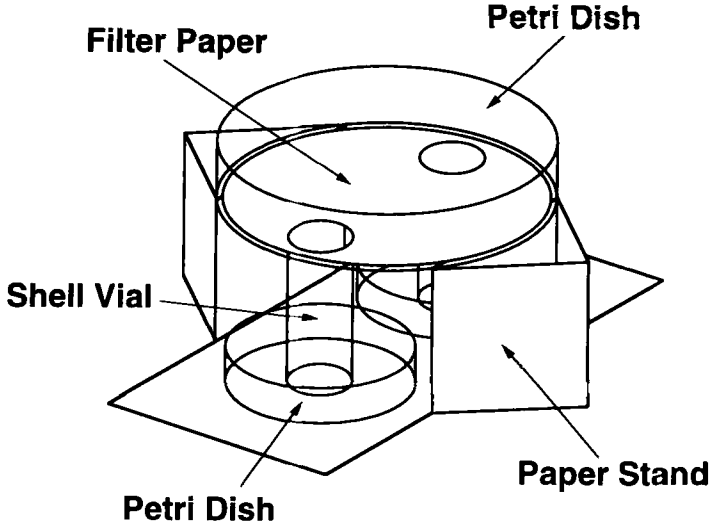


FIG. 1. Schematic of the two-choice bioassay apparatus and its five components, including three Petri dishes, two shell vials, filter paper lining, and a paper stand.

could still escape from between the arena and the dispensers, so each shell vial was set in a moat constructed from the bottom of a 60-mm × 15-mm-diameter Petri dish filled with water to capture them.

Compounds Tested. Dichloromethane, diethyl ether, acetone, hexane, and hexadecane were purchased from Mallinckrodt Specialty Chemicals (Swingey Ridge Rd., Chesterfield, Missouri 63017-1077). The diethyl ether was distilled over lithium aluminum hydride (LAH) to ensure dryness. Carbonated water (Canada Dry, Cadbury Beverages Inc., Stamford, Connecticut 06905-0800) served as a carbon dioxide source. Ethyl dichloroacetate was obtained from Pfaltz and Bauer Chemical Company (P.O. Box 5205, Flushing, New York 11352). Ethanol and benzene were obtained from Fisher Scientific (Fair Lawn, New Jersey 07410). Methanol was obtained from Alltech Associates, Inc. (Applied Science Labs, 2051 Waukegan Road, Deerfield, Illinois 60015).

Two-Choice Bioassay with Organic Solvents. Ten larvae were used in each replication. Larvae were taken randomly from our colony and transferred with a small, moist, camel-hair paint brush to the inside of a polystyrene cap from a 4-ml shell vial. Caps containing the larvae were kept on a moist paper towel to prevent the larvae from desiccating. The filter paper inside an arena was moistened with distilled water, and the excess was blotted away with a clean paper towel. The cap and the larvae were then placed into the center of the

arena and inverted. The cap was used to cover and contain the larvae there until commencement of the bioassay.

A 10-μl glass, fixed-needle syringe (Alltech Associate) was used to introduce 0.5, 1.0, 2.0, or 4.0 mg of distilled water, ethanol, methanol, acetone, ethyl dichloroacetate, dichloromethane, diethyl ether, benzene, hexadecane or hexane into the 4-ml glass shell vial on one side of the arena. The other dispenser remained empty. Both shell vial dispensers were positioned beneath the arena, the cap containing the larvae was removed, and the lid was placed over the petri dish arena. The duration of each bioassay was 30 min. When a replication was completed, the number of larvae in the bottom of the two dispensers and in the moat around them was recorded. WCR, SCR, and BCB larvae were used only once for testing, and each arena and dispenser was used only once. Temperature during the bioassays was between 23 and 27°C.

Two-Choice Bioassay with Carbonated Water. Carbonated water, used to test attraction of WCR larvae to different doses of carbon dioxide, was diluted and brought to a total volume of 200 μl with distilled water. Distilled water was introduced to the dispenser first with a 1-ml glass, fixed-needle syringe (Alltech Associates), and then the appropriate amount of carbonated water was added gently, also with a 1-ml glass, fixed-needle syringe (partial degassing of the carbonated water produced a headspace inside the syringe, and this headspace was not added). Ratios of carbonated water to distilled water used for our experiments were 0, 1:7, 1:3, 1:1, and undiluted carbonated water. For controls in our two choice tests, dispensers opposite the carbon dioxide treatments were filled with 200 μl distilled water. To assure a stable release of carbon dioxide and to prevent any effects from immediate out-gassing, 5 min were allowed to elapse between introduction of carbonated water to a dispenser and commencement of a bioassay. When both shell vials were prepared, behavioral bioassays were conducted as described above, except that 30 larvae were used for each replication and each bioassay was allowed to proceed for 10 minutes.

Statistical Analyses. Data were analyzed with SAS (SAS Institute Inc., SAS Circle, P.O. Box 8000, Cary, North Carolina 27512-8000). A t test was performed to identify any differences existing between the number of larvae attracted to the treatment dispenser and the control dispenser for each dose of all experiments. For the eluotropic series of compounds, ANOVA was conducted using differences between the number of larvae recovered from the treatment and control dispensers. Duncan's multiple range test was used to compare responses of WCR larvae to the compounds in the eluotropic series for each dose that yielded an ANOVA with a significant P value (0.05). Orthogonal comparisons of larval responses to increasing doses of each compound in the eluotropic series were made, identifying any linear or quadratic trends that may exist.

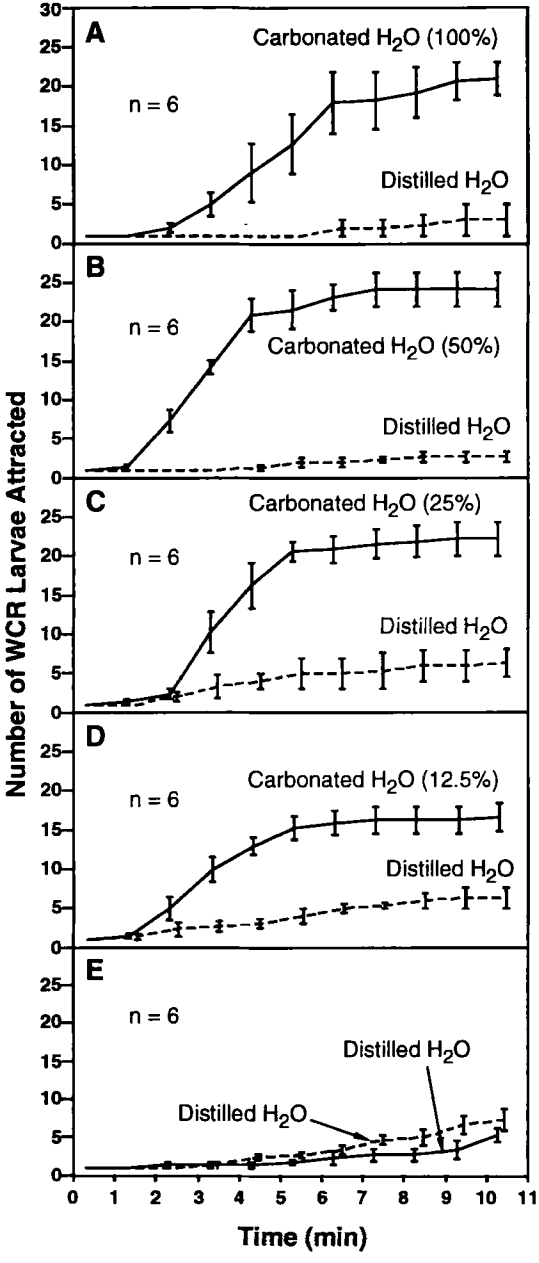
RESULTS

WCR Larvae. WCR larvae were attracted to carbon dioxide released from carbonated water (Figure 2). Carbon dioxide from the tested doses of carbonated water attracted significantly more WCR larvae after 10 min than controls of distilled water. Even the lowest concentration of carbonated water tested (12.5%) was significantly attractive. When WCR larvae were presented a choice of distilled water on both sides of the bioassay, they were recovered in equal numbers from both dispensers at its conclusion.

All doses of dichloromethane tested, including 0.5, 1.0, 2.0, and 4.0 mg, attracted second-instar WCR in our behavioral bioassay (Figure 3). A significantly greater number of WCR larvae were recovered from dispensers of dichloromethane than from empty dispensers. Orthogonal comparison of larval responses to increasing doses of dichloromethane revealed a quadratic (convex) trend. A significantly greater number of WCR larvae were recovered from dispensers of two benzene doses (0.5 and 2.0 mg) (Figure 3), but orthogonal comparison revealed no significant trend in larval responses to the doses tested. A significantly greater number of WCR larvae were also recovered from dispensers of one hexane dose (1.0 mg) (Figure 3), but orthogonal comparison revealed no significant trend in larval responses to the doses tested of that compound either. Although no significant attraction of larvae to any dose of methanol was observed, orthogonal comparison of larval responses to the tested doses revealed a significant (positive) linear trend. No significant attraction of WCR larvae to other solvents in the eluotropic series was observed (Figure 3), nor did orthogonal comparison reveal any significant trends in attraction of larvae to their increasing dose. Ethyl dichloroacetate was significantly repellent at all doses tested, which was expected because of its noxious qualities, and diethyl ether was repellent at three doses (0.5, 2.0, and 4.0 mg).

Multiple range tests revealed significant differences in the numbers of larvae attracted to each of the four doses among compounds in the eluotropic series.

FIG. 2. Behavioral responses of western corn rootworm (WCR) larvae *Diabrotica virgifera virgifera* to carbonated water (H_2O) as a carbon dioxide source. Each point on a line represents the average number of 30 larvae introduced to the center of the arena that were recovered from either of the two dispensers for n replications. Bars represent standard error values. (A) 100% carbonated H_2O = 200 μl of carbonated water introduced to the dispenser on the treatment side of the two-choice bioassay. (B) 50% carbonated H_2O = 100 μl of carbonated water + 100 μl of distilled water introduced to the dispenser on the treatment side. (C) 25% carbonated H_2O = 50 μl of carbonated water + 150 μl of distilled water introduced to the dispenser on the treatment side. (D) 12.5% carbonated H_2O = 25 μl of carbonated water + 175 μl of distilled water introduced to the dispenser on the treatment side. (E) Distilled H_2O = 200 μl of distilled water.


A
Carbonated H_2O (100%)
n = 6
Distilled H_2O
B
n = 6
Carbonated H_2O (50%)
Distilled H_2O
C
Carbonated H_2O (25%)
n = 6
Distilled H_2O
D
Carbonated H_2O (12.5%)
n = 6
Distilled H_2O
E
n = 6
Distilled H_2O
Distilled H_2O
Number of WCR Larvae Attracted
Time (min)
0 1 2 3 4 5 6 7 8 9 10 11
0 5 10 15 20 25 30

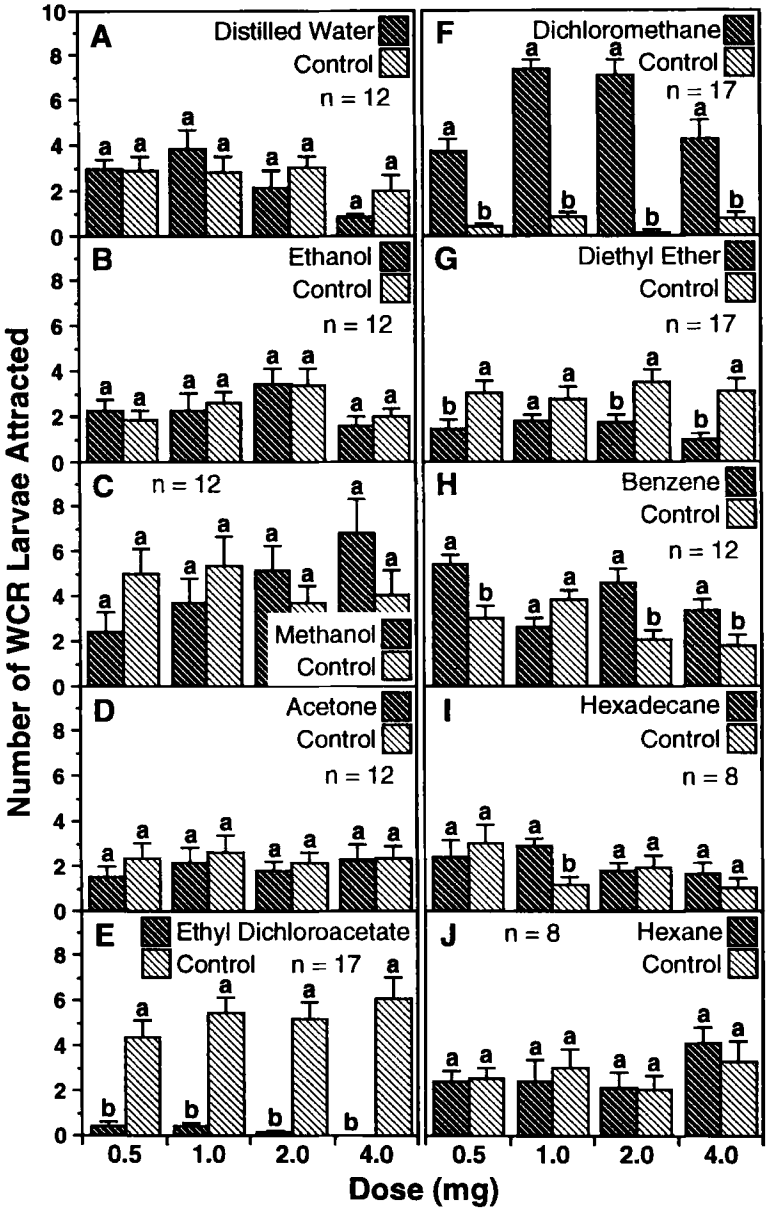


FIG. 3. Behavioral responses of western corn rootworm (WCR) larvae *Diabrotica virgifera virgifera* to an eluotropic series of compounds, including (A) distilled water, (B) ethanol, (C) methanol, (D) acetone, (E) ethyl dichloroacetate, (F) dichloromethane, (G) diethyl ether, (H) benzene, (I) hexadecane, and (J) hexane (0.5, 1.0, 2.0, and 4.0 mg); n = number of replications for each experiment. Bars on columns represent standard error values. Average responses to control and treatment sides within a dose denoted by the same letter are not significantly different ($P = 0.05$).

At the 0.5-mg dose, both dichloromethane and benzene were attractive and were not significantly different from one another. At the 1.0-mg dose, dichloromethane and hexadecane were the only compounds attractive to larvae, but dichloromethane was significantly more attractive than hexadecane. At the 2.0-mg dose, dichloromethane and benzene were the only compounds attractive to larvae, but dichloromethane was significantly more attractive than benzene. At the 4.0-mg dose, dichloromethane and benzene were the only compounds attractive to larvae, and they were not significantly different from one another.

SCR Larvae. SCR larvae were attracted to carbon dioxide released from carbonated water. When presented with a choice of 500 μl carbonated water and 500 μl distilled water, significantly more SCR larvae were attracted to carbonated water. With 17 replications, an average of 5.55 ± 0.68 larvae were recovered from in and around dispensers of carbon dioxide, while only 0.45 ± 0.15 larvae were recovered from in and around dispensers of distilled water. SCR larvae were also attracted to dichloromethane (Figure 4). Significantly more

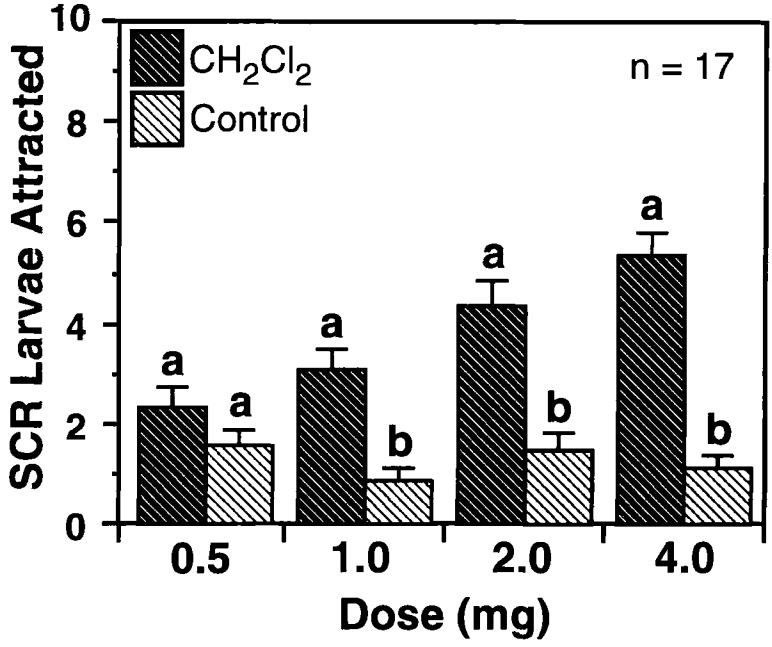


FIG. 4. Behavioral responses of southern corn rootworm (SCR) larvae *Diabrotica undecimpunctata howardi* to dichloromethane (CH_2Cl_2) (0.5, 1.0, 2.0, and 4.0 mg). There were 17 replications. Bars on columns represent standard error values. Average responses to control and treatment sides within a dose denoted by the same letter are not significantly different ($P = 0.05$).

SCR larvae were recovered from in and around dispensers of 1.0, 2.0, and 4.0 mg dichloromethane than from controls. The lowest dose of dichloromethane tested (0.5 mg) was not significantly attractive. Orthogonal comparison of SCR larval responses to increasing doses of dichloromethane revealed a significant (positive) linear trend.

BCB Larvae. BCB larvae were attracted to a 2.0-mg dose of dichloromethane. After eight replications in which BCB larvae were presented a choice of 2.0 mg dichloromethane and an empty dispenser, significantly more larvae were recovered from in and around dispensers of dichloromethane (4.13 ± 0.44) than from in and around empty dispensers (0.88 ± 0.23).

DISCUSSION

WCR larvae were attracted to carbonated water used as a carbon dioxide source, an observation consistent with that of Strnad et al. (1986). Dichloromethane also attracted second-instar WCR in our behavioral bioassay at all doses tested. These experiments represent the first time an organic compound has been demonstrated to attract WCR larvae in the absence of carbon dioxide. Dichloromethane also attracted second-instar SCR larvae for all but the lowest dose tested (0.5 mg) and elicited the attraction of BCB larvae at the only dose tested (2.0 mg). We also found that SCR larvae were attracted to carbonated water, which is the first report that carbon dioxide is an attractant for this species. The rationale for attraction of diabroticite larvae by carbon dioxide is its release from the roots of corn, a principal host (Massimino et al., 1980).

The selective attraction of WCR larvae to dichloromethane, but not to other organic solvents, may be a consequence of the mechanistic details of carbon dioxide reception by the insect. We suggest that carbon dioxide is oxidized to bicarbonate in the antennal hemolymph, and that bicarbonate is the form that is actually received by chemoreceptors in the dendrites. We further propose that dichloromethane mimics bicarbonate as it interacts with these chemoreceptors, and thereby results in larval attraction. As illustrated in Figure 5, the chlorine atoms of dichloromethane resemble the oxygen atoms of bicarbonate in size and electronegativity, and the bond angles between the heteroatoms are also similar.

Of the compounds in our eluotropic series, dichloromethane was the only one attractive at all doses tested. Most other solvents tested were neither attractive nor repellent, suggesting that the attraction to dichloromethane is not merely a response to organic solvents in general. These results also suggest that the general polarity of a compound is not an important component of its interaction with receptor sites on larvae, but that specific structural parameters are.

Many invertebrates of agricultural and medical importance, including the two diabroticite larvae discussed in this manuscript, cerambycid larvae (Meeking

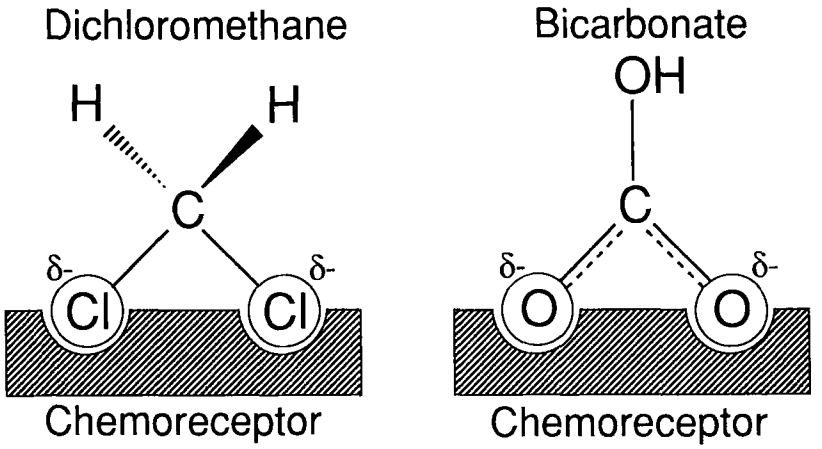


FIG. 5. Schematic of the model depicting mimicry between carbon dioxide (as bicarbonate) and dichloromethane at a molecular level in the context of receptor-semiochemical interaction.

et al., 1974), corn earworm larvae (Rasch and Rembold, 1994), mosquitos (Kellogg, 1970), tse tse flies (Bogner, 1992), stable flies (Warnes and Finlayson, 1986), tropical bont ticks (Steullet and Guerin, 1992; Perritt et al., 1993), and plant parasitic nematodes (Prot, 1980), are attracted by carbon dioxide. Furthermore, WCR, SCR, and BCB larvae are attracted by dichloromethane, which suggests that chemical mimicry of these compounds and other analogs may be common among invertebrates that use carbon dioxide for host-finding. If a relationship between carbon dioxide and dichloromethane is confirmed, new synthetic analogs may be available for use to enhance agricultural production or to reduce the incidence of invertebrate-spread diseases. Presently, because of the potential affect dichloromethane may have on the behavior of many invertebrates that use carbon dioxide for host-finding, behavioral results obtained after using it to extract, isolate, and identify semiochemicals should be regarded critically.

Its migratory habits have rendered attempts to manage the adult stage of the WCR unsuccessful, so management strategies have concerned the larvae (Levine and Oloumi-Sadeghi, 1991). Protection of corn roots has relied primarily upon soil insecticides (Suguiyama and Carlson, 1985), but the need for an alternative management strategy has been expressed because of concerns for resistance, the environment, and the potential for extended diapause (Stoewen, 1989). In response to this need, semiochemicals have been included as part of integrated pest management programs (Bjostad et al., 1993), and the goal of developing more convenient formulations with an increased range of activity

has inspired researchers to consider synthetic analogs of semiochemicals (Briggs et al., 1986).

Halogenated analogs of semiochemicals are behaviorally active for other insect species. (*E*8,*E*10)-dodecadien-1-ol (codlemone) is the main sex pheromone component of the codling moth, *Cydia pomonella*, and halogenated analogs were tested for field activity with the expectation that they may be more effective than the native form (Lucas et al., 1994). F(10,11)-codlemone and Cl-codlemone were more attractive to male codling moths than codlemone itself. A series of mono-, di-, and trihalogenated methyl-substituted analogs of (*E*8,*E*10)-dodecadien-1-yl acetate, a sex attractant for the alfalfa moth, *Cydia medicaginis*, were less active than the original acetate (Svatos et al., 1990). Many structure–activity studies with semiochemicals have considered the replacement of hydrogen atoms on moth pheromones with fluorine atoms (Camps et al., 1984; McLean et al., 1989; Prestwich et al., 1986; Bengtsson et al., 1990; Linn et al., 1992). Analogs of this nature were still active, but insects usually required a longer recovery time. Other structure–activity studies have considered optically active analogs (Suzuki et al., 1984; Li et al., 1986), and methyl-substituted frontalins (Payne et al., 1988).

Semiochemicals could facilitate management of the WCR in many ways. Use of baits with insecticides could reduce the amount of insecticides applied to manage populations of WCR larvae in the field. Soil insecticides do not necessarily control populations of larvae, but instead protect root systems from injury by confining damage to peripheral roots or ones outside the protected band [Felsot et al. as cited in Levine and Oloumi-Sadeghi (1991)]. Current application procedures of corn growers appear to be compatible with semiochemical techniques for enhancing the efficiency of insecticides (Hibbard et al., 1994). WCR soil insecticides are typically formulated on clay, sand, or gypsum carrier granules (Levine and Oloumi-Sadeghi, 1991), and semiochemicals could be formulated with them to develop an attracticide. Semiochemicals may encourage feeding of WCR larvae on artificial diets to facilitate screening of insecticides and improve laboratory rearing techniques (Sven Strnad, American Cyanamid, Box 400, Princeton, NJ 08540-0400, personal communication). Use of artificial diets has already improved rearing of the SCR in colonies and facilitated screening of insecticides (Marrone et al., 1985).

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REPRODUCTIVE PERFORMANCE AND POPULATION DYNAMICS OF COWPEA APHID (Homoptera: Aphididae) ON LEAF EXTRACTS OF RESISTANT AND SUSCEPTIBLE COWPEAS

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Abstract—Membrane feeding studies were conducted to determine the effects of raw juices and chemical extracts of leaves of aphid-resistant (ICV-12) and aphid-susceptible (ICV-1) cultivars of cowpea [*Vigna unguiculata* L. (Walp.)], on the survival, growth, and reproduction of cowpea aphid *Aphis craccivora* Koch. Life table and demographic statistics of the cohort population and subsequent generations were estimated. Compared to ICV-1, the leaf juices and chemical extracts of ICV-12 exhibited significant ($P \leq 0.05$) adverse effects on aphid survival, growth, and reproduction. Raw leaf juice and ethyl acetate extract of ICV-12 in both water and sucrose significantly ($P \leq 0.05$) limited aphid performance. The adverse long-term effects were often more extreme than those resulting from a diet of distilled water alone. Methanol extract of ICV-12 showed an intermediate level of adverse effects on aphids, being generally less than that of ethyl acetate but greater than that of hexane. Compared to the other ICV-12 extracts, the hexane extracts in water or sucrose media did not significantly affect the aphid performance. Overall, it was determined that antibiosis was a governing modality of aphid-resistance in ICV-

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12. Postingestive intoxication was caused by foliage components of seedling plants of that cultivar.

Key Words—Net reproduction rate, postingestive intoxication, membrane feeding, ethyl acetate extract, sucrose, ICV-12, antibiosis, aphid performance *Vigna unguiculata*, *Aphis craccivora*, cowpea resistance, cowpea aphid.

INTRODUCTION

Effects of certain phytochemicals and different cowpea cultivars on aspects of the biology of the cowpea aphid, *Aphis craccivora* Koch have been well studied (Mansour et al., 1982; Lattanzio et al., 1990). Extensive research using electronic feeding monitoring has been conducted on aphid feeding behavior on different crops and cultivars of the same crop (McLean and Kinsey, 1964; Nielson and Don, 1974; Brown and Holbrook, 1976; van Helden and Tjallingii, 1993; Prado and Tjallingii, 1994), and on plant extracts and other artificial diets (Tjallingii, 1985; Annan, 1992). However, the effects of plant extracts on aphid reproduction and population dynamics have not been well-documented.

Cowpea *Vigna unguiculata* (L.) Walp., is an important crop throughout the tropics, due to its nutritional and agronomic value (Singh and Rachie, 1985). *Aphis craccivora* is the most serious insect pest of the cowpeas worldwide (Singh et al., 1978). It attacks and damages all parts and growth stages of the plant. Genetic studies and selections of aphid-resistant cowpea germplasm have been extensively studied (Bata et al., 1987; Ombakho et al., 1987; Pathak, 1988), but identification of the specific mechanisms of aphid resistance in cowpeas is not well understood.

The cowpea cultivars selected for these studies were the aphid-susceptible cv. ICV-1 and the aphid-resistant cv. ICV-12, both of which were registered and released by International Center of Insect Physiology and Ecology (ICIPE) in Kenya (Pathak and Olela, 1986). ICV-1 was a single-plant selection developed from landraces of eastern Kenya, whereas ICV-12 was obtained from ICV-1 by point mutation of a chromosome using gamma irradiation. Both cultivars have similar morphological habits and phenological traits, but differ mainly in their resistance or susceptibility to *Aphis craccivora*. Aphid resistance in ICV-12 is highly expressed in seedling plants (Pathak, 1988; Annan, 1992).

This study was conducted as part of an overall research effort to characterize the mechanisms of the observed field resistance of ICV-12 against *Aphis craccivora*. Previous studies using electrical penetration graphs from aphid feeding behavior on seedling plants and leaf extracts of ICV-12 suggested that antixenosis is a governing mechanism of aphid resistance in that cultivar (Annan, 1992). Other studies with plants and excised plant parts of that cultivar indicated

that seedling plants of ICV-12 expressed high levels of antibiosis against the reproductive performance and population dynamics of the *A. craccivora* (Annan, 1992; Givovich et al., 1988; Firempong, 1988).

The main objective of this work was to evaluate the role of chemical extracts from ICV-12 seedling foliage in modulating the antibiosis resistance in that cultivar, using the aphid-susceptible ICV-1 cultivar as a standard check. Horizontal life tables (Southwood, 1978) were used to analyze the aphid performance and demographic statistics on the two crop cultivars.

METHODS AND MATERIALS

Laboratory and greenhouse studies in this work were conducted at the ICIPE-Mbita Point Field Station in Western Kenya. There were four blocks corresponding to the timing of experiments, namely: May to June 1990, August to September 1990, November to December 1990, and February to March 1991.

Experimental Design and Test Conditions. The test was set up in a randomized complete block design, with six replications of each treatment in each block. Experiments were conducted in the laboratory at 26 ± 0.5°C, 40–80% relative humidity, and supplied by an overhead bank of 40-W Tezla Z Daylight fluorescent lamps (Tezla, Czech Republic). Aphids used were the progenies of a colony obtained with a few field-collected individuals that were maintained in the greenhouse on plants of a susceptible cowpea cultivar VITA-7 at 25 ± 2°C, 40–90% relative humidity and a 14L:10D photo period. Fresh plants were added to the colony every three days, to maintain colony vigor.

Raw leaf juices and solvent extracts from aphid-resistant ICV-12 and aphid-susceptible ICV-1 cultivars were used to study the expression of cowpea resistance to *A. craccivora* in terms of the effects on aphid reproductive performance. Plant extracts dissolved in either water or in 0.5 M sucrose solution were compared to a double control system consisting of sucrose solution only, on which there is high aphid feeding and survival, and to plain water, on which aphid feeding and survival is low.

In order to ascertain that the three solvents used in the extractions were not toxic to the aphid, a 100-μl titer of each solvent was homogenized in plain water or sucrose solution and tested on aphids. The experiment was repeated five times. It was first done before tests with plant extracts and was repeated as a set of control treatments in all four tests with plant extract tests.

Overall there were 16 treatments: eight plant extracts consisting of hexane extracts, ethyl acetate extracts, methanol extracts, and raw leaf juices of each ICV-12 and ICV-1; and eight control treatments consisting of distilled water only or 0.5 M sucrose solution or either solution with 100 μl aliquots of the respective solvents added.

Leaf Sample Collection and Extraction Method. Field grown plants were used in the studies. Foliage of 2-week-old seedling plants of cv. ICV-12 and cv. ICV-1 were harvested. Samples were carefully handled to maintain plant integrity. Prior to extraction, leaf samples were frozen in a portable ice chest.

Extraction of leaf samples was started within 30 min after harvesting. Sequential extractions of leaves were made with hexane, ethyl acetate, and methanol, in order of increasing solvent polarity. Extracts were obtained by soaking 50 g fresh foliage in 150 ml of each solvent for 24 hr. All extractions were done at room temperature. After extraction with each solvent, leaves were air-dried at room temperature to remove solvent residues. Leaf fresh weights were recorded before extraction and after extraction with each solvent. Percent fresh weights of leaf matter extracted in the different solvents were calculated.

After each extraction, solvent residues in the extracts were evaporated with a Büchi model RE-111 Rotavapor Evaporator (American Scientific, McGaw Park, Illinois). Then the extracted matter was redissolved in 100 ml of the same solvent to obtain the stock solution used for each test. Prior to bioassay, 100 μl of an extract were homogenized in 1 ml of water for ca. 10 min using a hand-operated Fischer Sonic Dismembrator Portable Homogenizer (Fischer Scientific, Pittsburgh, Pennsylvania).

Membrane Feeding Bioassay. Cylindrical plastic vials (7 cm long $\times$ 3 cm diam.), with a small hole (1 cm diam.) cut at the top end and the bottom end sealed, were used as test units. Each vial consisted of two 3.5-cm-long cylindrical plastic drums joined in the middle with a 32-mesh/mm^2 No-See-Um organdy screen. Two holes (0.5 $\times$ 0.5 cm) were cut on opposite sides of the bottom drum of the vials beneath the organdy screen to improve ventilation, and vials were covered with black muslin tape to exclude light.

During the membrane feeding tests, homogenates were dispensed into feeding membrane sachets made of sterilized finely stretched 2-cm $\times$ 2-cm pieces of Parafilm M (American National Can, Greenwich, Connecticut) and placed over the hole at the top of the test vial. Five apterous adult aphids of similar age were placed on the organdy screen and provided feeding access to parafilm sachets with the test solutions. Vials were placed in small cups half-filled with water to exclude ants. Each vial comprised a treatment replicate. Determination of aphid stylet penetration of sachets during feeding was made with a Wild light microscope (50–60 Hz) (Wild Co., Heerbrugg, Switzerland).

Data Collection, Parameters, and Analyses. Daily observations of aphids were made, and new nymphs or dead individuals were counted and removed. Data were recorded for each vial until all cohort individuals died. In a few cases, vials were discarded due to leakage of sachets, causing mortality and colony collapse. Specific data used to estimate aphid life table statistics included: survival to adult stage (l_x), net rate of reproduction (R_O), intrinsic rate of natural increase (r_m), and number of generations. Data analyses were performed using

ANOVA (SAS Institute, 1988). Data on the percentage of surviving aphids covered a wide range of values. Therefore, those data were subjected to arcsin transformations (Snedecor and Cochran, 1980). Significant differences in treatment effects of similar extracts of ICV-12 and ICV-1 or the different extracts of each cultivar were determined using 95% confidence intervals and orthogonal contrasts.

RESULTS

Calculations of mean percent fresh leaf weights of ICV-12 that was extracted in the various solvents were: 33% weight in hexane, 21% in ethyl acetate, and 16% in methanol, with 30% being the unextracted leaf matter; for ICV-1 there was 31% weight in hexane, 16% in ethyl acetate, 12% in methanol, and 41% remaining in the residue after extractions. Initial inspection of the aphid life table data indicated similar trends in distribution. Thus, there was no significant effect of blocking or time of experimentation, and the raw data for all four experiments were pooled and analyzed.

At the 95% CI, there were no significant differences in the aphid population parameters on plain water only compared to when the respective solvents were mixed with water. Similarly, there were no significant differences between aphid performance on plain water diet and 0.5 M sucrose diet plus solvents (Table 1). However, specific orthogonal contrasts indicated that all aphid life table param-

TABLE 1. SUMMARY OF COWPEA APHID LIFE TABLE DATA[a]

Diet	Survival l_x[b]	Intrinsic rate of increase, r_m	Net reproduction rate, R_0	Generations (N)
Water	0.43 ± 0.001	0.22 ± 0.001	8.5 ± 1.02	1.2 ± 0.05
Water + hexane	0.61 ± 0.18	0.25 ± 0.05	6.5 ± 1.72	0.9 ± 0.12
Water + ethyl acetate	0.38 ± 0.07	0.31 ± 0.12	6.8 ± 0.92	1.0 ± 0.11
Water + methanol	0.48 ± 0.09	0.18 ± 0.06	6.3 ± 1.4	0.9 ± 0.20
Sucrose	0.93 ± 0.02	0.55 ± 0.06	82.6 ± 5.1	1.8 ± 0.28
Sucrose + hexane	0.77 ± 0.23	0.60 ± 0.09	70.1 ± 12	1.6 ± 0.02
Sucrose + ethyl acetate	0.82 ± 0.21	0.48 ± 0.11	66.2 ± 9.8	1.9 ± 0.38
Sucrose + methanol	0.97 ± 0.10	0.44 ± 0.08	88.4 ± 17	1.4 ± 0.33

[a] Based on diet treatments of distilled water or 0.5 M sucrose solution only, and where 100 μl of hexane or ethyl acetate or methanol are added to either medium (means ± 95% CI).

[b] Proportion of cohort individuals surviving to adult stage to reproduce; arcsin transformed data (N = 30)

eters estimated were significantly reduced ($F_{1,60} = 4.03$, $P \leq 0.05$) on plain water diet compared to the sucrose diet. Those two treatments were used as the control checks in comparing effects of the various leaf extracts of the two cultivars.

ANOVA revealed that in comparison with the respective controls of water or sucrose diets, there were significant effects of ICV-12 extracts on aphid survival to the adult stage for both extracts in water ($F_{8,207} = 27.04$, $P < 0.001$) and extracts in sucrose ($F_{8,211} = 43.56$, $P < 0.001$) (Figure 1). Furthermore, there was a decreased number of generations for extracts in sucrose ($F_{8,211} = 10.98$, $P < 0.001$) (Figure 2).

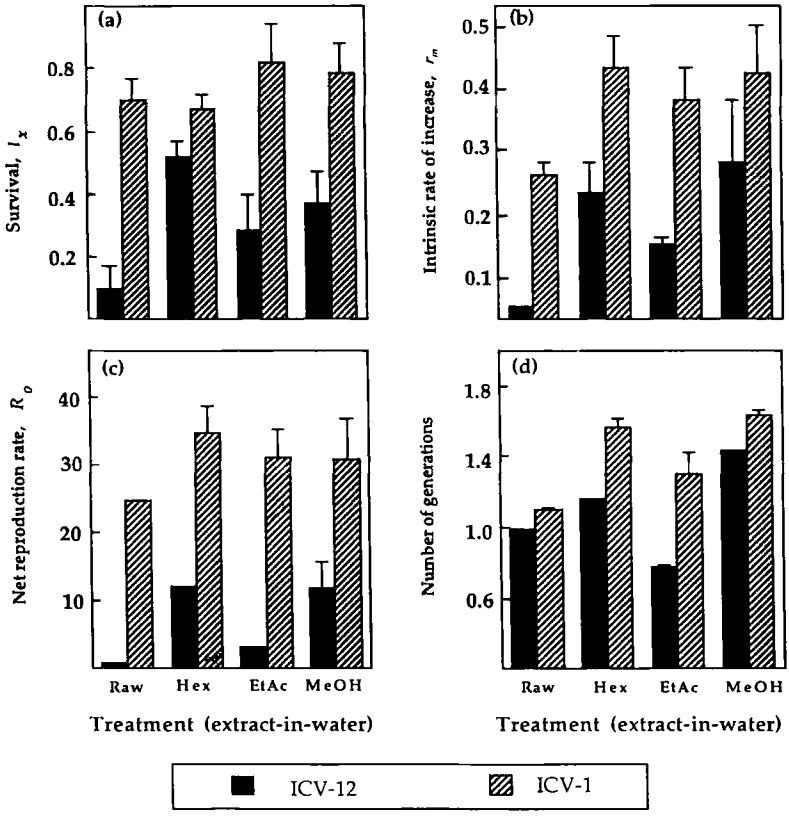

FIG. 1. Interactions between cowpea cultivar and treatments of leaf extracts in water, on cowpea aphid life table parameters: (a) survival l_x, (b) intrinsic rate of increase r_m, (c) net reproduction rate R_O, and (d) number of generations, (means ± 95% CI; $N = 24$).

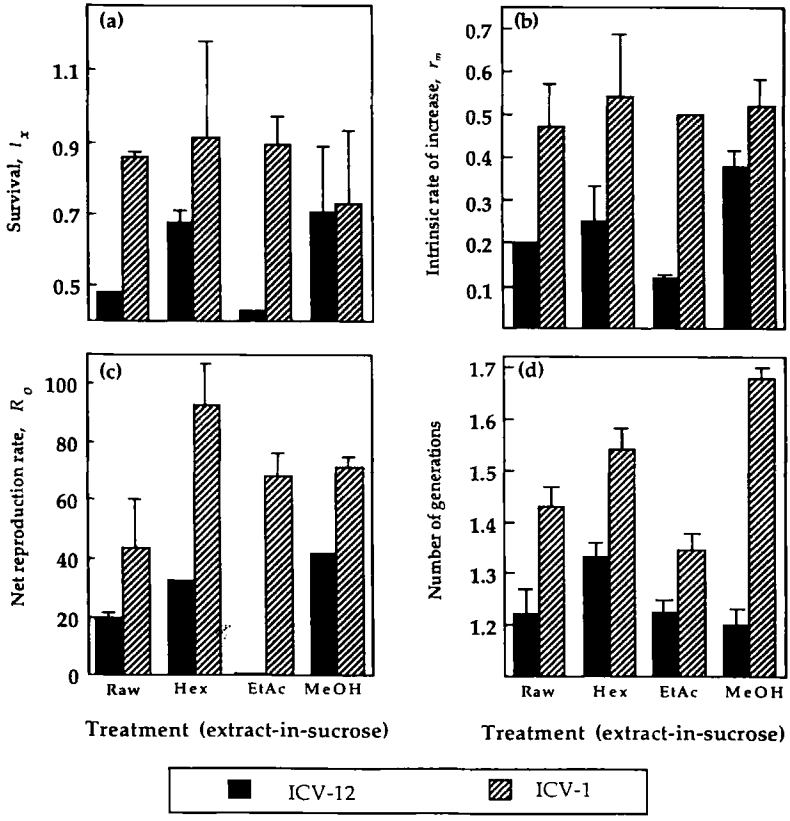

FIG. 2. Interactions between cowpea cultivar and treatments of leaf extracts in sucrose, on cowpea aphid life table parameters: (a) survival l_x, (b) intrinsic rate of increase r_m, (c) net reproduction rate R_O, and (d) number of generations; (means ± 95% CI; N = 24).

Compared to similar extracts of ICV-1 foliage, the ethyl acetate extract and raw juice of ICV-12 significantly impaired all life table parameters of cowpea aphid. Compared to the ICV-1 methanol extracts, the same fractions of ICV-12 had variable activity. ICV-12 methanol extract in water significantly limited aphid survival and net reproductive rate (Figure 1), while the same extract in sucrose reduced the intrinsic rate of increase and net reproduction rate (Figure 2). For ICV-12, the ethyl acetate extract and raw leaf juice in both water and sucrose media generally reduced cowpea aphid performance to a greater extent than did the hexane and methanol extracts. The exceptions were in water, where activity of the ethyl acetate extract was not significantly different

from the methanol extract activity for aphid survival to adult stage, and in sucrose, where the raw leaf juice activity did not significantly differ from the hexane extract for intrinsic rate of increase. Furthermore, activity of both ethyl acetate extract and raw leaf juice of ICV-12 in sucrose were not significantly different from the methanol extract for the number of generations.

Thus, the long-term adverse effects of ICV-12 extracts on aphid reproduction and growth performance were greater than those of ICV-1. The levels of reduction in aphid performance by both the ethyl acetate extract and raw leaf juice of ICV-12 were not mitigated in sucrose medium, but in the cases of net reproduction rate and number of generations produced, the effects were even more severe.

DISCUSSION

Cowpea aphid survival, growth, and reproductive biology were adversely affected by ethyl acetate extracts and raw leaf juices of the aphid-resistant ICV-12 cultivar, indicating the presence of unidentified chemical resistance factors in the foliage of that cultivar. Antibiosis activity of the ethyl acetate fraction was pronounced and was not reduced in the sucrose medium. This suggested the possibility of feeding deterrence activity in that fraction. However, using electrical penetration graphs of *A. craccivora* feeding behavior, Annan (1992) determined that individual aphids readily fed on ICV-12 leaf extracts, suggesting that the resistance factor would also most likely act as a postingestive intoxicant. Hence, the author concluded that the observed field aphid resistance in the ICV-12 cultivar was governed by both antixenosis and antibiosis. This assertion corroborated the findings of Givovich et al. (1988) and Firempong (1988) with *A. craccivora* feeding on the same cowpea cultivar. Similar studies and findings of resistance to other aphids species in various crop cultivars also have been well-documented (Niassy et al., 1987; van Helden and Tjallingii, 1993; Tjallingii, 1985; van Helden, 1995).

In comparison to other solvents, ethyl acetate extracts contain many phenolics and other secondary compounds, whereas methanol extracts contain mainly glycosides and other sugar derivatives. Hexane extracts contain mainly nonpolar materials such as leaf waxes, fatty acids, and cholorophyll (J. A. A. Renwick, personal communication). The possible involvement of flavonoids, phenols, and coumarins from the ICV-12 ethyl acetate extract in impairment of cowpea aphid performance is consistent with previous reports on that cultivar and other resistant cowpea cultivars by MacFoy and Dabrowski (1984), Lattanzio et al. (1990), and Mansour et al. (1982). While we did not determine the exact components of the ethyl acetate extracts, the high activity of that fraction, coupled with the findings in those previous studies on the chemical composition of similar extracts from cowpeas, suggested the involvement of phenolics in the aphid resistance

of ICV-12. None of the fractions of ICV-1 extract produced any significant impairment of aphid performance in either water or sucrose medium, nor did any of the various ICV-1 extracts differ from each other.

Although the sucrose diet was used as a control treatment to ensure maximal aphid feeding, continual rearing of aphid generations on it was not possible, perhaps because the diet lacked other nutrients that are essential for optimal survival, growth, and reproduction of aphids. When compared to bioassays on intact plants and excised plant parts reported elsewhere (Annan, 1992), there were sharp declines in net reproduction rate and intrinsic rate of increase of aphids. This can be partially attributed to the lack of other essential nutrients in the sucrose diet. Nevertheless, since sucrose diet provided better nutrition and thus enhanced aphid performance than just plain water in earlier tests (Table 1), it was a suitable medium for the plant extracts.

Leaf waxes were implicated in aphid resistance in barley (Tsumuki et al., 1989). However, due to the lack of activity of the hexane extract, which should have extracted leaf waxes, chlorophyll, and fatty acids, and since the ethyl acetate extracts that produced deleterious effects on aphid performance would not contain waxes and other leaf surface materials, we suggest that those factors probably do not contribute to aphid resistance in ICV-12. Similarly, we would argue that glycosides and other factors extracted in the methanol extracts also do not contribute significantly to aphid resistance in that cultivar.

Seedling foliage of both varieties is already extensively used for human consumption and in livestock feeds in East Africa and other tropical regions. The seedling leaf factors that were extracted in the ethyl acetate fraction and that were involved in ICV-12 impairment of *A. craccivora* performance could therefore have specific aphicidal properties, but little or no toxic effects to humans or livestock. However, both size and the relative amounts of leaf material ingested are likely to be potential factors in toxicity.

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INVOLVEMENT OF STRICTOSIDINE AS A DEFENSIVE CHEMICAL IN *Catharanthus roseus*

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Abstract—The involvement of the glucoalkaloid strictosidine in antimicrobial and antifeedant activity in *Catharanthus roseus* leaves was investigated. Strictosidine and its deglucosylation product, specifically formed by the enzyme strictosidine glucosidase, were shown to be active against several microorganisms. In contrast, neither the intact glucoside, nor the aglycone product(s) was found to exhibit antifeedant activity against *Spodoptera exigua* larvae, as was found for intact *C. roseus* leaves and leaf extracts. Besides alkaloids further downstream in the biosynthesis pathway, a more apolar, yet unidentified compound may be involved in this activity.

Key Words—Antifeedant, fungi, bacteria, *Spodoptera exigua*, *Catharanthus roseus*, *Fusarium solani*, *Fusarium oxysporum*, *Trichoderma viride*, *Cladosporium cucumerinum*, *Phytophthora infestans*, strictosidine, strictosidine glucosidase, indole alkaloids.

INTRODUCTION

Madagascar periwinkle *Catharanthus roseus* (L.) G. Don (Apocynaceae) is a plant of pharmaceutical interest since it produces a number of important sec-

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ondary metabolites used in medicine. For example, the dimeric alkaloids vinblastine and vincristine are invaluable in the treatment of acute lymphatic leukemia (DeConti and Creasey, 1975; Neuss and Neuss, 1990).

The reason why the plant produces these compounds is still unclear. It has been suggested that these chemicals function as defenses against herbivores. Indeed, antifeedant activity against polyphagous *Spodoptera* larvae has been reported for *C. roseus* extracts (Chockalingam et al., 1989), and for the alkaloids vinblastine and catharanthine (Meisner et al., 1981). The reported induction of biosynthesis of these indole alkaloids in *C. roseus* after moderate wounding (Frischknecht et al., 1987) supports such a defense mechanism. Alkaloids from *C. roseus* have also been reported to show antimicrobial activity. For example, ajmalicine, catharanthine, and vindoline appeared to inhibit bacterial and fungal growth (Hernandez, 1979). These compounds may therefore be multifunctional in their biological role. However, it is not evident whether the antifeedant and antimicrobial effects of *C. roseus* extracts are solely caused by the mentioned alkaloids; more compounds might be involved in this activity.

In this paper, the involvement of strictosidine (**1**; Figure 1) in the chemical defensive system of *C. roseus* was studied. This glucoalkaloid is considered to

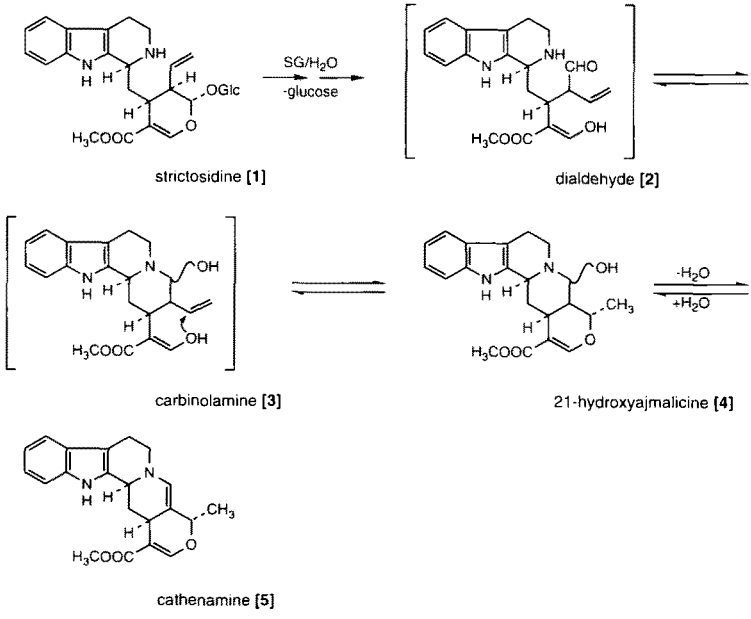

FIG. 1. Scheme showing the conversion of strictosidine and subsequent steps in *C. roseus*.

be the general precursor of all monoterpene indole alkaloids (Stöckigt and Zenk, 1977). It is formed by condensation of secologanin with tryptamine in a reaction catalyzed by the enzyme strictosidine synthase (SSS; EC 4.3.3.2). It has been shown that this reaction takes place inside the cell vacuole (McKnight et al., 1991). The occurrence of strictosidine itself can be confirmed in extracts of the plant (particularly in young leaves), suggesting the existence of a vacuolar pool. The converting enzyme, strictosidine glucosidase (SG; EC 3.2.1.105), is suspected to be located on the outside of the vacuolar membrane (Stevens et al., 1993). In this way, the substrate can be deglucosylated only if this barrier is overcome, either when transported over the vacuolar membrane or when the membrane is actually damaged.

The hypothesis we tested is that the strictosidine pool inside the cell vacuole serves in a "trigger mechanism" in which cell damage leads to strictosidine deglucosylation, resulting in the formation of compound(s) with antimicrobial and/or antifeedant activity. Concentration of SG on the vacuolar membrane guarantees a high turnover rate of the substrate. Strictosidine deglucosylation leads, via a number of reactive intermediates (Stöckigt et al., 1978), to 21-hydroxyajmalicine (**4**; see Figure 1) (Stevens, 1994).

A similar trigger mechanism has been reported for *Tulipa* (Beijersbergen and Lemmers, 1972), and for oat (*Avena sativa*) leaves. In the latter, vacuolar glucosides were shown to be involved in a mechanism in which deglucosylation by a specific tonoplast avenacosidase yields aglycones with a strong antifungal activity (Kesselmeier and Urban, 1983; Grünweller and Kesselmeier, 1985). In our paper, the involvement of strictosidine and SG in the antifeedant activity of *C. roseus* leaves was investigated. For comparison, the activity of constitutive compounds further downstream in the indole alkaloid biosynthetic pathway was investigated by using *C. roseus* leaf extracts. As test organisms, larvae of beet army worm, *Spodoptera exigua* (Noctuidae), were used since species of this genus are generally polyphagous; they have proven to be useful for judging antifeedant activity of alkaloids (Meisner et al., 1981; Chockalingam et al., 1989).

Antimicrobial activity of strictosidine and its turnover products was investigated by in vitro incubation of several species of plant fungi and bacteria in different concentrations of strictosidine (both with and without SG).

METHODS AND MATERIALS

Chemicals were from Merck, except for *p*-methylhydroxybenzoate, ascorbic acid, tryptamine hydrochloride, and secologanine (Aldrich), and vancomycine (Duchefa, Haarlem, The Netherlands). Organic solvents were distilled prior to use. The secologanin obtained was further purified over a silica gel column

by using a stepwise acetone gradient (0–50%) in ethyl acetate. Strictosidine was synthesized by condensation of secologanin with tryptamine catalyzed by purified strictosidine synthase from *C. roseus*, immobilized on a column of CNBr-activated Sepharose (Pharmacia) (Pfitzner and Zenk, 1982).

C. roseus plants were grown in a greenhouse at 20°C and 70% humidity, in a 16-hr/8-hr day–night regime. Ten plants were used to prepare a leaf extract by grinding 11.8 g of freshly picked leaves in 200 ml of methanol–HCl 0.02 M (1:1, v/v), using an Ultra-Turrax. A part of this extract was lyophilized. This dry extract was used to prepare different concentrations of extract in methanol for testing. Alkaloids were determined in the extract by HPLC with photodiode array detection (van der Heijden et al., 1987). A linear gradient of acetonitrile (15–50%) was the eluent. Quantification of strictosidine was made from an external standard. Catharanthine, vindoline, and anhydrovinblastine were quantified from absorbances at their respective UV maxima, using molar absorption coefficients reported in the literature (Hesse, 1964, 1968).

The SG used was partially purified from *C. roseus* cell cultures (Luijendijk, unpublished data). SG activity was determined using the HPLC assay described by Stevens et al. (1993). SG stability was tested by incubating a part of the enzyme solution at 25°C for 24 hr. Regularly a sample was taken and frozen at −80°C. The collected samples were then assayed for SG activity.

Test Organisms. Larvae of *Spodoptera exigua* (Lepidoptera: Noctuidae) were reared on an artificial diet consisting of maize grains (224 g), brewer's yeast (60 g), wheat germ (56 g), agar (40 g), ascorbic acid (9.6 g), benzoic acid (2.4 g), methyl-*p*-hydroxybenzoate (2 g), and vancomycin (50 mg), prepared in 1360 ml of water. Larvae were kept at 23°C and 70% relative humidity in a controlled 16-hr/8-hr day–night regime. Larvae were used when they reached the L4 stage (approx. 10 days after hatching), in order to assure good feeding and accurate weight measurement. The larvae were offered leaves of winter purslane (miner's lettuce), *Claytonia perfoliata* Willd., a vegetable not known to produce alkaloids, that had been treated with different amounts of test substances. This vegetable was chosen because of its suitability as food for *Spodoptera* larvae and its relatively flat leaves, facilitating quantification of herbivory by leaf area measurements. Single larvae were starved for 3 hr, weighed, and transferred to separate Petri dishes containing one test leaf per dish together with a moistened piece of filter paper. The *C. perfoliata* leaves were treated with 100 μl of test solution (Figure 2) and dried at room temperature to allow evaporation of the solvent (methanol) before feeding the leaves to the larvae. In the control series E and F, leaves were treated with solvent only. For each concentration of test substance, 10 Petri dishes were used. For determination of antifeedant activity, larval weight increase and leaf area decrease after 24 hr and 48 hr were measured. For the measurement of leaf areas, a video-operated

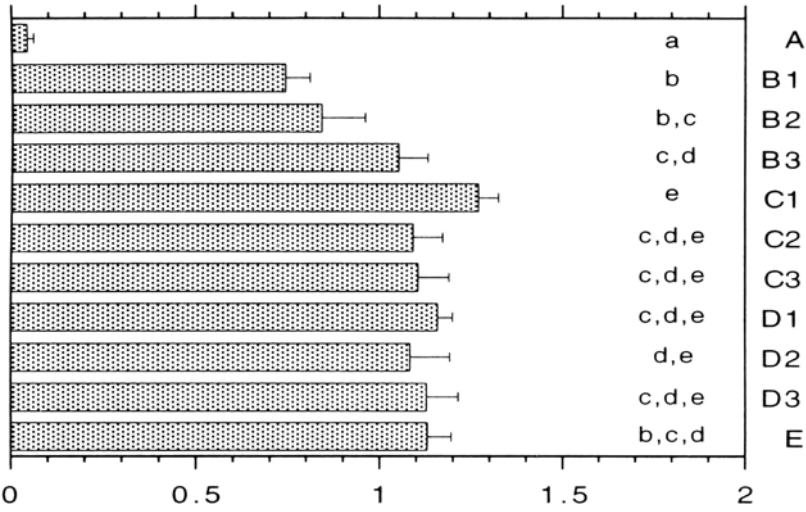

FIG. 2. Preference indices for *Spodoptera exigua* feeding for 48 hr on leaves ($N = 10$ per series) treated with the following test substances:

Series	Foliage	Test Solution
A	*Catharanthus roseus*	none
B1	*Claytonia perfoliata*	*C. roseus* leaf extract (5× conc)
B2	*Claytonia perfoliata*	*C. roseus* leaf extract (undiluted)
B3	*Claytonia perfoliata*	*C. roseus* leaf extract (5× diluted)
C1	*Claytonia perfoliata*	200 μg strictosidine
C2	*Claytonia perfoliata*	40 μg strictosidine
C3	*Claytonia perfoliata*	4 μg strictosidine
D1	*Claytonia perfoliata*	as C1, with 100 μl SG sol. (0.13 nKat) applied to the other side of the leaf
D2	*Claytonia perfoliata*	as C2, with 100 μl SG sol. (0.13 nKat) applied to the other side of the leaf
D3	*Claytonia perfoliata*	as C3, with 100 μl SG sol. (0.13 nKat) applied to the other side of the leaf
E	*Claytonia perfoliata*	100 μl SG solution (0.13 nKat)

Error bars indicate the standard errors per group. The lowercase letters a, b, c, d, e indicate the categories in which the test series could be grouped according to a multiple range analysis.

planimeter was used. After 48 hr, the larvae were frozen in liquid nitrogen and extracted with methanol by homogenization with a glass rod.

In a different set-up, analogous to that described by Kubo (1991), larvae were offered a choice of pieces of artificial diet prepared with *C. roseus* extracts.

For this purpose the original methanol–hydrochloric acid extract was freeze-dried and redissolved in (A) 0.1 M NaOH or (B) 0.1 M HCl, and extracted using dichloromethane. The antifeedant effects of these extracts and of the aqueous layers after extraction were compared with those of the original methanol–hydrochloric acid extract, a leaf extract prepared with petroleum ether (boiling range 60–80°C), and a control without additions.

Test media were prepared in small portions by adding the extract to one of the constituents of the artificial medium and subsequent evaporation of the solvent, before preparation of the medium.

A total of 49 larvae (seven per plate) was offered the choice between seven disks (diameter 8 mm) of these diet media. Four times during 24 hr (after 1, 5, 12, and 24 hr), the diet preference of the larvae was assessed by counting larvae on the different disks.

For the antifungal activity tests, *Fusarium solani*, *Fusarium oxysporum*, and *Trichoderma viride*, originally isolated from fruits (Department of Phytopathology, Wageningen), and *Cladosporium cucumerinum* (CBS 177.54) were grown on potato dextrose agar. *Phytophthora infestans*, originally isolated from potato (Department of Phytopathology, Wageningen), was grown on rye agar. The bacterial strains *Escherichia coli* (ATCC 8739) and *Bacillus subtilis* (ATCC 6633) were maintained in CASO broth. All media mentioned were obtained from Merck.

All dilutions and inoculations were carried out under sterile conditions. Fungal tests were performed in 24-well or 96-well microtiter plates with 250 μl or 50 μl of potato dextrose agar, respectively. Per well, approx. 600 sporangia (in 50 μl) were added to the 24-well plates, and approx. 150 sporangia (in 25 μl) to the 96-well plates. Germination was allowed for 6 hr at 25°C, after which 100 μl of test solution was added to each well (50 μl for the 96-well plates). Test solutions were prepared in 50 mM potassium phosphate buffer (pH 6.0) (15 mM phosphate, with 20 mM sodium chloride for *Phytophthora infestans*), and sterilized by filtration through 0.2-μm cellulose acetate filters (Schleicher & Schüll). Buffer exchange of the SG preparations was performed by gel filtration over Sephadex G-25 (PD-10 columns, Pharmacia). For most species, a concentration range of 0.003–25 mM of strictosidine was tested in duplicate (nine concentrations, four wells per concentration) and compared to a similar range of strictosidine concentrations in the presence of SG (75 pKat, 1 Kat defined as the enzyme activity converting 1 mole of strictosidine per second, at 30°C). As controls, similar tests were done using preincubated strictosidine–SG mixtures, SG solution alone, and inactivated SG solution to exclude the possibility of the enzymes solution itself being active on microbial growth. Control of inhibition was done with nystatin (1 mg/ml).

Comparative incubations were done with ajmalicine and yohimbine. It was necessary to use a solubilizing agent for ajmalicine; Tween 80 in a concentration of 0.25% was found to solubilize the compound sufficiently without itself having

any influence on fungal growth. The preparations were sterilized by autoclaving for 20 min (121°C, 1 bar overpressure). Ajmalicine proved to be stable during this procedure. All incubations were done in the dark at 25°C until good growth was obtained in the control wells (usually after 72 hr). Growth was judged visually before and after staining the mycelia with lactophenol cotton blue. Minimum inhibitory concentration values were determined from the lowest concentrations in the wells not showing fungal growth. For more detailed investigation a Nikon binocular microscope was used.

Tests with bacteria were carried out using liquid media (CASO broth) in glass test tubes. In a total volume of 260 μl (125 μl CASO, 100 μl strictosidine in aqueous solution, 25 μl = 75 pKat SG solution, 10 μl inoculum), bacteria were incubated in the presence of a range of strictosidine concentrations (0.05–0.5 mM). For each concentration tested, three tubes were used. After 16 hr of incubation at 37°C, growth was determined by the turbidity of the solutions.

RESULTS AND DISCUSSION

Alkaloid contents found in fresh *C. roseus* leaves are summarized in Table 1. Noteworthy is the relative low content of strictosidine compared to the compounds further downstream in the biosynthetic pathway. This is in accordance with earlier results, which showed that strictosidine in indole alkaloid-producing plants is mainly formed in the young leaves, while later products are predominantly found in older leaves (Aerts et al., 1991; Naaranlahti et al., 1991). SG activity of the tested solution was found not to be affected by incubation at 25°C, since no activity loss was observed, even after 24 hr. Therefore, it was concluded that the enzyme was stable enough to be used in the tests for biological activity.

Antifeedant Tests. Figure 2 shows the preference indices (PI) derived from the antifeedant tests after 48 hr, in which $PI = 2T/(C + T)$. In this formula,

TABLE 1. CONTENT OF SOME INDOLE ALKALOIDS IN MATURE AND YOUNG *C. roseus* LEAVES[a]

	Mature leaves		Young leaves	
	μmol/g FW	μg/g FW	μmol/g FW	μg/g FW
Strictosidine	0.12 ± 0.06	63 ± 29	0.50 ± 0.06	260 ± 30
Catharanthine	0.79 ± 0.10	260 ± 30	2.32 ± 0.12	780 ± 40
Vindoline	0.45 ± 0.05	200 ± 20	n.d.	n.d.
Anhydrovinblastine	1.03 ± 0.07	820 ± 60	n.d.	n.d.

[a]Averages (and standard deviations) are given for three extractions of 40 leaves each. (n.d. = not determined)

C is the leaf area eaten in the control, and T is the area eaten from the test leaves. Thus, a PI = 0 implies a strong deterrent activity of the test substance, PI = 1 no preference, and PI = 2 a strong rejection of the control or preference for the test substance (Kogan and Fischer, 1991). The data were normally distributed in each group. No toxic effects of the applied test solutions were suspected, since only two of the larvae died during the experiment.

A one-way analysis of variance (ANOVA) of the PI values demonstrated significant differences in the antifeedant activity between the tested groups ($\phi = 11$, $F = 19.24$, $P < 0.0001$). To test which groups differed from each other, a multiple-range analysis was used to distinguish between homogeneous groups (Figure 2). Group A (*C. roseus* leaves) was significantly different from all other groups, and group B1 (the highest concentration of *C. roseus* leaf extract) was different from all others but group B2. The average activity in the leaf extract preparations (B1, 2, and 3) seemed to follow the applied concentration. The activity of these *C. roseus* leaf constituents was accentuated by the fact that the highest applied amount of leaf extract (B1) accounted for the contents of only 10% of an average *C. roseus* leaf, as calculated from the leaf area.

The results presented here confirmed the presence of antifeedant compounds in *C. roseus* leaves, since the acidic methanol extract already showed a significant activity. The involvement of strictosidine, however, seems to be unlikely. Both the series with strictosidine solution applied alone and the ones with strictosidine and SG combined showed no significant antifeedant activity, even though the highest concentrations (C1 and D1) were more than an order of magnitude higher than the concentrations in vivo in old leaves, and three times higher than in young leaves. The proposed trigger mechanism, in which the active compound is only formed by enzymatic conversion after plant damage, is therefore not likely to be an important factor in the plant's defense against this insect herbivore. A chemical turnover of the aglycone product during the test procedure cannot be excluded, but is unlikely since cathenamine is reported to be stable and precipitates on formation (Stevens, 1994). There is the possibility that the tested compounds might show effects towards other insect species less polyphagous than the *Spodoptera* species tested here.

No strictosidine could be retrieved from the larvae fed with strictosidine and SG combined, so an inactivation of SG by the feeding larvae or by the plant material is not likely. Strictosidine itself was probably not affected by nonspecific decomposition since significant amounts could be retrieved by methanol extraction of the larvae fed with leaves treated with strictosidine alone (results not shown). Apparently the larvae were not harmed by this compound, which was retrieved in concentrations up to 30 μg/g body weight.

Experiments in which the test extracts were incorporated in the rearing medium of the *Spodoptera* larvae showed that the active compounds in the methanol–hydrochloric acid extract were largely present in the alkaline dichloromethane preparation made from this extract, indicating an alkaloidal nature.

However, the other fractions obtained from this preparation were not preferred by the larvae either; the untreated preparation was significantly favored (Kruskal-Wallis test, $P < 0.05$). Not only the MeOH–HCl extract showed a pronounced antifeedant activity, but so did a petroleum ether leaf preparation (Figure 3). Since alkaloids are too polar to be extracted with petroleum ether, the involvement of other active compounds is suspected. This suggests the presence of antifeedant compounds of a very apolar nature, which might therefore be present in the wax layer of the leaves, thus presenting a chemical barrier against herbivores. The findings that *C. roseus* leaves were mostly left untouched by the tested larvae and the antixenotic (as opposed to antibiotic) nature of the components support this hypothesis. Alkaloids are predominantly found deeper down in the *C. roseus* mesophyll since idioblasts were found to contain a significantly higher alkaloid concentration (Mersey and Cutler, 1986), making these products less likely to be responsible for this result. Further research into the apolar fraction may give more clarity in this respect.

Antimicrobial Tests. After 72 hr of incubation, the higher concentrations of tested substances showed clear growth inhibition. Growth was normal in the controls. Any influence by the enzyme preparation itself, or by the buffer used,

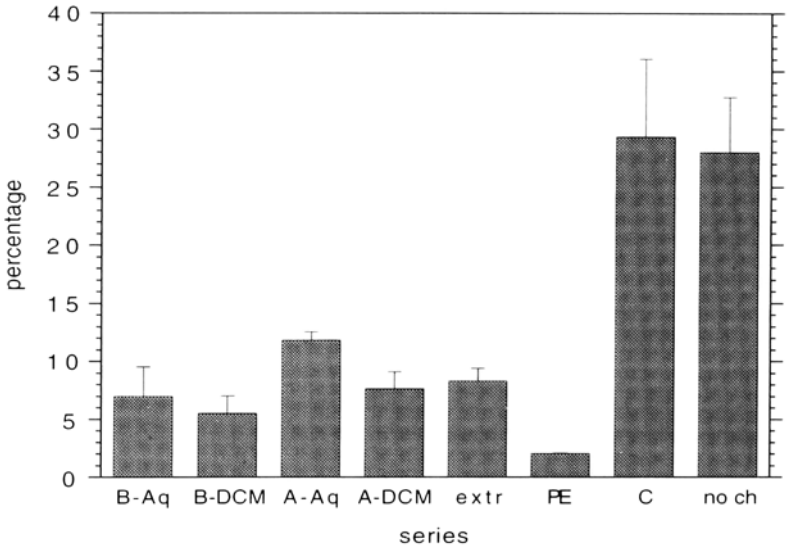

FIG. 3. Percentage of *Spodoptera exigua* larvae present on seven pieces of feeding media prepared with different *C. roseus* extracts. Average values and standard errors are shown for four time points during 24 hr. B-Aq = basic aqueous fraction after DCM extraction, B-DCM = basic DCM extract, A-Aq = acidic aqueous fraction after DCM extraction, A-DCM = acidic DCM extract, extr = original acidic MeOH leaf extract, PE = petroleum ether leaf extract, C = control, no ch = no choice.

TABLE 2. MINIMUM INHIBITORY CONCENTRATION OF STRICTOSIDINE AND STRICTOSIDINE IN COMBINATION WITH STRICTOSIDINE GLUCOSIDASE (SG) TO SEVERAL FUNGAL SPECIES

Species	Strictosidine (mM)	SG/strictosidine (mM)
Fusarium solani	25	0.33
Fusarium oxysporum	25	0.33
Trichoderma viride	10	1
Cladosporium cucumerinum	>1	0.33
Phytophthora infestans	<0.008	<0.008

could thus be excluded. In all cases fungal growth was inhibited in the presence of nystatin. From Table 2 a clear difference can be seen in the minimum inhibitory concentration (MIC) values of strictosidine compared to those of strictosidine that was converted by SG. For several species, the MIC value of strictosidine/SG was around 0.33 mM. Only *Phytophthora infestans* showed a different pattern; even the slightest addition of strictosidine inhibited development, suggesting a strong antifungal activity of strictosidine against this phytopathogen. On the other hand, this activity may not necessarily be specific, since *Phytophthora infestans* has been reported to be very susceptible to differences in culture conditions (Woloshuk et al., 1991).

Microscopic study of the fungal material in the other species present in concentrations just below the MIC value showed that hyphae were thinner and showed no or only very few divisions, as compared to those in controls. This might suggest an action on the developing hypha membrane or cell wall, causing a lethal lysis. No sporangicidal effects by the tested substances could be determined.

Comparison of incubations of strictosidine with SG in situ, or with SG before addition to the developing fungi (thus allowing a complete turnover of strictosidine to 21-hydroxyajmalicine), showed marked differences; this suggests this product to be the active compound. However, it is possible that an equilibrium exists in the rearrangements of the aglycone after SG-mediated hydrolysis, providing a sufficient concentration in situ of one of the intermediates for expression of its antifungal activity.

Although the MIC value of 0.33 mM (180 μg/ml) may not be as low as those for some other antifungally active alkaloids, e.g., cryptopleurine (0.3 μM; Al-Shamma et al., 1982), it may indicate an antifungal function in vivo, since strictosidine concentrations in young *C. roseus* leaves of 260 μg/g fresh weight and SG levels of 750 pKat/g were found. In contrast, older *C. roseus* leaves were found to contain significantly lower levels of both strictosidine and SG. The young leaves, vulnerable during their development, may very well be using these high levels of strictosidine as a protective mechanism.

For comparison, similar assays were performed using two other corynan-

thean type alkaloids, yohimbine and ajmalicine, as test substances. No influence on fungal growth was observed in the concentration range used (0.008–0.83 mM). This is an indication that the antimicrobial activity found in the strictosidine/SG assays is not related to the general structure of the corynanthean type of indole alkaloids but is more specifically caused by 21-hydroxyajmalicine (**4**) or cathenamine (**5**) formed after strictosidine deglucosylation.

Bacterial assays showed growth inhibition of *B. subtilis* for concentrations over 0.1 mM strictosidine in the presence of SG, while strictosidine alone did not show any effect. *E. coli* was only inhibited at the highest concentration tested (0.5 mM strictosidine, with SG), again with no effect in incubations with strictosidine alone. It thus represented a similar situation to that described in most antifungal tests. Therefore, an antimicrobial compound seems to be involved that is formed only after deglucosylation of strictosidine.

CONCLUSIONS

Strictosidine, and especially its aglycone product, were shown to possess antifungal activity in vitro. The compounds may therefore be involved in an antimicrobial defense system in indole alkaloid-producing plants. In particular, the turnover product, after deglucosylation by SG, shows activity at physiological concentrations. The subcellular separation of strictosidine formation and conversion (by SG) in *C. roseus* may serve a trigger mechanism against such infections. On the other hand, this defensive system does not seem to be involved in the antifeedant activity *C. roseus* leaves show against *Spodoptera exigua* larvae. In addition to an acidic methanol extract, a petroleum ether extract of *C. roseus* leaves also showed antifeedant activity, suggesting an apolar nature of some active compound(s). The indole alkaloids present in the leaves of *C. roseus*, reported in previous studies to show antifeedant potential, may therefore not be the only compounds responsible for the in vivo activity.

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COMBINED CHEMICAL DEFENSES AGAINST AN INSECT–FUNGAL COMPLEX

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Abstract—This study considered how host plant allelochemicals may contribute to defense against insects and fungi that jointly colonize the subcortical tissues of trees, the relative roles of constitutive and inducible chemistry in these defenses, and how the actions of two different feeding guilds might be interrelated. Our model consisted of the coniferous tree *Pinus resinosa*, the root- and lower stem-colonizing beetles *Hylastes porculus* and *Dendroctonus valens*, and their associated fungi *Leptographium procerum* and *L. terebrantis*, and the stem-colonizing bark beetle *Ips pini* and its associated fungus *Ophiostoma ips*. In a novel bioassay, extracts from reaction tissue elicted by wound inoculation with *L. terebrantis* were more repellent to beetles than were similar extracts from constitutive or mechanically wounded tissue. The effect on beetle behavior was more pronounced in nonpolar extracts, which contain mostly monoterpenes, than in polar extracts, which contain mostly phenolics. Synthetic monoterpenes at concentrations present in the various tissues exerted similar effects and were likewise repellent in dose–response experiments. Growth of *L. procerum* and *L. terebrantis* was inhibited by polar extracts from constitutive and reaction tissue. Inhibition was higher in wounded than control tissue, but the inhibition response did not vary with the type of wounding. Synthetic monoterpenes strongly inhibited spore germination and mycelial growth of both fungi. Colonization of red pine roots by *Leptographium* spp. altered the subsequent effects of extracts of stem phloem tissue on *I. pini*. These effects varied with host condition. Beetles preferred extracts from constitutive stem phloem tissue of healthy trees to that of root-diseased trees. However, extracts from reaction tissues of healthy trees were more repellent

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to *I. pini* than were the reaction tissues of root-diseased trees. The implications of these results to plant defense against insect-fungal complexes and interactions among different feeding guilds are discussed.

Key Words—Insect-fungal interactions, host selection, plant stress, forest decline, monoterpenes, phenolics, bark beetles, *Ophiostoma ips*, *Ips pini*, *Dendroctonus valens*, *Hylastes porculus*, *Leptographium procerum*, *Leptographium terebrantis*, *Pinus resinosa*.

INTRODUCTION

Plant parasitic insects and plant pathogenic microorganisms are confronted with similar defensive mechanisms when invading potential host plants (Schultz, 1983; Krischik et al., 1991; Hammerschmidt, 1993); yet the chemical ecology of plant defense against these organisms has largely been studied separately. Recent discussions of plant defense theory emphasized the need for integrated studies on plant-insect-microbial interactions (e.g., Barbosa et al., 1991; Schowalter and Filip, 1993). Bark beetle (Coleoptera: Scolytidae)-ophiostomatoid fungi complexes that colonize living conifers provide a valuable system for comparing host plant defenses against herbivory and disease. Most bark beetle species vector only a few, or at most several, ophiostomatoid wood-colonizing fungi. These insect-fungal symbioses may initially be mutually advantageous in overcoming host tree resistance (Berryman, 1972; Hemingway et al., 1977; Raffa and Berryman, 1983a; Berryman et al., 1985; Whitney, 1982), but fungi may reduce beetle success during subsequent development (Barras, 1970; Bridges and Perry, 1985; Fox et al., 1983).

The physiology and chemistry of conifer constitutive and induced defenses against attack by phloem-invading complexes have received significant attention. Constitutive defenses include tough outer bark, several classes of allelochemicals, and elaborate networks of resin ducts or glands. Attacks by invading bark beetles elicit localized host responses including rapid formation of resinous lesions around the attack sites (Berryman, 1972; Raffa and Smalley, 1995). In instances of successful tree defense, the beetle and its associated microbes are contained within these lesions. When the tree is unsuccessful, the beetle and microbes colonize and kill host tissue. In these cases, host defensive reactions are only barely apparent and appear to have been exhausted by the rapid arrival of numerous beetle-fungal units mediated by aggregation pheromones (Raffa and Berryman, 1983a,b).

Two classes of allelochemicals, monoterpenes and phenolics, are particularly abundant in conifer subcortical tissue and increase in concentration in response to invasion of living phloem (Jorgensen, 1961; Shain, 1967; Russell and Berryman, 1976; Raffa and Berryman, 1982, 1983b; Hain et al., 1983;

Cook and Hain, 1986; Faeth, 1986; Lorio, 1986; Miller et al., 1986; Paine et al., 1987; Lewinsohn et al., 1991; Herms and Mattson, 1992; Klepzig et al., 1995b). Hence, these compounds have been proposed as important components of chemical defense. Evidence in support of this hypothesis includes observations that monoterpenes can inhibit mycelial growth of beetle-vectored fungi and repel or kill bark beetles under some conditions (Smith, 1963; Cobb et al., 1968; Shrimpton and Whitney, 1968; DeGroot, 1972; Coyne and Lott, 1976; Bordasch and Berryman, 1977; Raffa et al., 1985; Bridges, 1987; Delorme and Lieutier, 1990; Paine and Hanlon, 1994). However, we know little about how host defenses may affect beetle and fungal establishment during the early and most critical phases of this interaction. In particular, integrated studies on how host chemistry affects fungal propagule germination and beetle host-selection behavior are lacking.

We have utilized an insect–fungal complex associated with a forest decline disease to investigate chemical defenses in a coniferous host plant (Klepzig et al., 1991, 1995a,b). This complex includes root-infesting (*Hylastes porculus* Erichson) and stem-infesting (*Dendroctonus valens* LeConte) bark beetles (Scolytidae) that vector two ophiostomatoid fungi, *Leptographium terebrantis* Barras and Perry and *L. procerum* (Kendrick) Wingfield, to red pine (Klepzig et al., 1995a). Colonization by this complex is not lethal to mature red pine. The pine engraver, *Ips pini* (Say) (Scolytidae), colonizes the stem above ground, vectors the fungus *Ophiostoma ips* (Rumb.) Nannf., and ultimately kills declining trees (Klepzig et al., 1991).

In this study we sought to determine the role of monoterpenes and phenolics in the constitutive and inducible defenses of red pine against two insect–fungal complexes from different feeding guilds and examine the effects of previous activity by one feeding guild on subsequent plant-defense reactions against another.

METHODS AND MATERIALS

Two sets of experiments were conducted. The first series evaluated the effects of constitutive and induced allelochemicals on subcortical beetles and their associated fungi. Healthy, mature red pines were wound-inoculated with *L. terebrantis* to simulate natural attacks. Phloem tissue was collected from these inoculations at one and three weeks after inoculation to determine if the host reaction changed with time since inoculation. Extracts from constitutive (unwounded) and reaction (wound-inoculated) phloem tissue were tested in insect behavioral, fungal germination, and fungal growth assays. The monoterpene and phenolic compositions of these extracts were previously reported in Klepzig et al. (1995b). The bioassays were also conducted using defined concentrations

of synthetic monoterpenes that simulated the composition of host constitutive and reaction tissue. The tests with synthetic monoterpenes included both individual compounds and combinations, as recent work demonstrates the importance of synergistic interactions in some systems (e.g., Berenbaum et al., 1989).

A second set of experiments considered how colonization by a member of a root-colonizing guild (*L. terebrantis*, the fungal associate of *H. porculus*) affected host plant interactions with a member of a stem-colonizing guild (*I. pini*). Our previous research demonstrated an association between colonization by *I. pini* and prior root infection with *Leptographium* spp. (Raffa and Klepzig, 1996). In the current set of experiments, the effects of extracts from healthy and root-diseased trees on behavior of stem-colonizing beetles were compared.

Insect and Fungal Culture

Adult *H. porculus* and *D. valens* were collected from red pine plantations and held at 7°C until use. Adult *I. pini* were obtained from a laboratory colony reared in red pine logs using previously described methods (Raffa and Smalley, 1995). Refrigerated slant cultures of *L. terebrantis* (isolate K9013, from an adult *D. valens*, and isolate LT23, from a *Pinus resinosa* Ait. root) and *L. procerum* [isolate LP5, obtained from an adult *Hylobius radicis* Buchanan (Curculionidae)] were transferred to plates of potato dextrose agar (PDA) and allowed to grow for 10–14 days at 24°C.

Host Treatment and Extraction

Healthy, mature red pines were wound-inoculated (Wright, 1933) to the phloem–xylem interface with a 12-mm disk of PDA colonized by *L. terebrantis*, mechanically wounded with a sterile 12-mm cork borer, or left unwounded (constitutive). Reaction lesions were sampled at one or three weeks after inoculation. In the wounding and inoculation treatments, the xylem and phloem tissues containing the resinous reaction lesion were removed with a chisel and transported to the laboratory on Dry Ice. Samples of constitutive tissues of similar size were removed from a randomly chosen site at the same height on the stem, but were otherwise treated the same as wounded and inoculated tissues. The phloem was separated from the xylem and weighed. The phloem was chopped into small ($< 1 \times 1$ mm) pieces and extracted for 24 hr at 25°C, in either polar or nonpolar solvents. Polar solvents were used to extract phenolics. In 1986, tissue samples were extracted in 10 ml of 50% methanol for 24 hr at 25°C. In 1993, tissue samples were extracted in 10 ml of 70% acetone for 24 hr at 25°C. Extracts were filtered through medium-porosity filter paper and stored at −30°C in sealed vials. Constitutive and reaction tissues were extracted in 10 ml of pentane for 24 hr at 25°C to extract monoterpenes. Extracts were

filtered through medium-porosity filter paper and stored at −30°C in sealed vials.

Effects of Induced and Constitutive Allelochemicals on Beetle Host Selection Behavior

Polar extracts (primarily phenolics), nonpolar extracts (primarily monoterpenes), or synthetic pure monoterpenes (Klepzig et al., 1995b) were incorporated into artificial media.

Polar Extracts. A host selection medium was developed to elicit tunneling and feeding behaviors. Melted 1.5% PDA (500 ml) was combined with 80 g of ground, freeze-dried, autoclaved, constitutive red pine phloem from healthy unwounded mature trees. A strip of plastic transparency film was sealed with melted paraffin wax across the middle of a half-section of a two-compartment I-plate Petri dish (Fisher Scientific, Pittsburgh, Pennsylvania). Test extracts were added directly to the two quarter-sections of the Petri plate. A 0.5-ml aliquot of one extract was added to one section of the assay medium, and 0.5 ml of a different extract was added to the other section, such that the resulting concentrations within the medium were similar to those found in an equivalent weight of red pine phloem. Molten host selection medium (15 ml) was mixed thoroughly with the extracts on each side and allowed to solidify, after which the plastic divider was removed. This assay was used to compare the biological acitivity of polar extracts from unwounded phloem tissue and reaction phloem tissue that had been wound-inoculated with *L. terebrantis* and sampled after three weeks on the tunneling behavior of *D. valens*, *H. porculus*, and *I. pini*. Polar extracts from mechanically wounded tissue were not available for use in these assays.

Nonpolar Extracts. A modified medium was prepared from freeze-dried, ground red pine phloem, water, and Bacto-agar (Difco, Detroit, Michigan) 2:0.002:1 (phloem–water–agar). Approximately 1.5 g of medium was pressed into a 5-mm layer within one of the half-sections of a two-compartment I-plate Petri dish, allowed to dry, and divided into two quarter-sections with a razor blade. Nonpolar extracts from constitutive, mechanically wounded, or inoculated trees were concentrated from 2.0 to 0.5 ml under nitrogen. Subsamples (0.25 ml) of these extracts were added to one or the other quarter-section of medium. The pentane was evaporated under nitrogen, and the razor blade divider was removed.

This assay was used to compare the biological activity of nonpolar extracts from unwounded phloem tissue, phloem tissue that had been mechanically wounded and sampled after three weeks, and phloem tissue that had been wound-inoculated with *L. terebrantis* and sampled after one or three weeks. Preliminary assays of the effects of these extracts on *H. porculus* and *I. pini* tunneling

behavior (Klepzig, 1994) revealed no significant differences between extracts of 1-week-old and 3-week-old inoculations. In addition, these two types of extracts were not found to differ significantly in monoterpene composition (Klepzig et al., 1995b). Due to this, and to the availability of extracts, extracts from 3-week-old inoculations were compared to extracts from constitutive phloem, and extracts from 1-week-old inoculations were compared to extracts from 3-week-old mechanical wounds. *D. valens* was only sporadically available during this time period and was excluded from further assays. However, because *H. porculus* was readily available from the field, and a laboratory colony of *I. pini* was readily available during this time period, these two species were used in this assay.

In all of the above assays, a semicircular piece of plastic transparency film was placed on top of the medium and sealed in place with molten paraffin wax. A hole (2–3 mm in diameter) was drilled through the midpoint of the half-section divider, and 4 mm into the medium. The plate was tilted and either one female *D. valens* or *H. porculus*, or one male *I. pini* (the host-selecting sex, respectively) was introduced into the hole. The hole was sealed behind the insect with cotton and transparent tape. The beetle could then enter either of the two quadrants and move freely between them. The lid of each dish was replaced and sealed with Parafilm. All assay chambers were incubated in a dark growth chamber at 24°C and examined daily for three to five days. Each beetle's path was traced, and the distance tunneled was recorded daily. The location of the beetle was also recorded for each assay dish. For purposes of this assay, the beetle was considered to have made no choice between the two extract types if any part of its body lay along the midline separating the two sections of medium. If, at the time of measurement, the beetle was entirely within one of the two sections, it was recorded as being located on that side for that day. For this reason, the percentages of time on either side of the assay chamber often did not add up to 100%. The total amount of tunneling and the percentage of time located in each side were analyzed by ANOVA (Abacus Concepts, 1989). Where significant treatment effects were observed, the means were compared using the protected least squares means procedure.

Synthetic Monoterpenes. Two-way choice assays were used to compare the effects of concentrations of α-pinene, camphene, β-pinene, 3-carene, myrcene, and limonene equivalent to those found in unwounded, mechanically wounded, and wound-inoculated mature red pine phloem (Klepzig et al., 1995b) on tunneling by *I. pini*. *I. pini* was used because of availability from a laboratory colony and also because of its pivotal role as a final mortality agent in red pine decline disease (Klepzig et al., 1991).

The monoterpenes tested comprise approximately 99% of the monoterpene fraction of red pine phloem (Raffa and Smalley, 1995). We calculated the amount

of each monoterpene necessary (milligrams per gram of medium) to simulate the concentrations (milligrams per gram phloem) found in constitutive, mechanically wounded, and wound-inoculated phloem tissues (Klepzig et al., 1995b). Each section of medium (3.65 g) meant to simulate constitutive phloem received only 0.004 ml of α-pinene. All the other major monoterpenes occurred at levels too low to incorporate effectively into the medium. Each section of host selection medium meant to simulate mechanically wounded phloem sampled after three weeks received 0.066 ml α-pinene, 0.022 ml β-pinene, 0.002 ml 3-carene, and no camphene, myrcene, or limonene. Each section of host selection medium meant to simulate *L. terebrantis* wound-inoculated phloem sampled after three weeks received 0.168 ml α-pinene, 0.001 ml camphene, 0.068 ml β-pinene, 0.005 ml 3-carene, 0.001 ml myrcene, and 0.002 ml limonene. The effects of α-pinene and β-pinene on tunneling by *I. pini* were also compared to each other in two-way choice assays using the concentrations of these compounds described above.

The effects of increasing concentrations of α-pinene and β-pinene on *I. pini* feeding were examined in no-choice assays. In one series of assays, beetles were introduced into host selection medium containing 3.4, 6.8, 17.1, 34.3, 171.6, or 343.2 mg α-pinene/g of medium. A separate series of assays was conducted using the same range of concentrations of β-pinene. Mean amounts of tunneling were measured and analyzed as described above.

Effects of Induced and Constitutive Allelochemistry on Fungal Germination and Growth

The effects of host allelochemicals on fungal growth and germination were determined by exposing spores and/or mycelia to polar host extracts and/or synthetic monoterpenes.

Fungal Propagule Germination. The effects of monoterpenes on fungal germination were determined using the synthetic monoterpenes described above. Molten PDA (0.05 ml) was dispensed into the wells of sterile Falcon 96-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, New Jersey), and allowed to solidify. Spore suspensions (200,000 spores/ml) were prepared from actively growing colonies of *L. terebrantis* or *L. procerum* and dispensed in 0.005-ml aliquots into each well. Sterile glass fiber filter paper disks (0.7 cm diameter) were placed so that they fit snugly in the top of each well but did not touch the agar surface 0.5 cm below. Each of the common red pine monoterpenes was assayed at saturated concentrations (200 μl applied to each filter paper disk) against both fungi. Varying concentrations of α-pinene and β-pinene were also tested. A total of 12 assay wells were tested per fungus–monoterpene combination. Plates were incubated, with lids in place, at 20°C in darkness. Mean percentages of germination were calculated for each fungus–monoterpene com-

bination, and compared using the protected least squares means procedure in ANOVA (Abacus Concepts, 1989).

Fungal Growth. Polar extracts from phloem of mature red pine trees (N = 25) that had been left unwounded, aseptically mechanically wounded, or wound-inoculated with actively growing *L. terebrantis* were incorporated into PDA. The crude extracts were first partitioned in chloroform, and the residue was dissolved in 40 μl of a 1 : 1 acetone–water mixture. Molten PDA (1 ml) was dispensed into the vial, the vial was capped and agitated, set horizontally until the agar solidified, and uncapped to allow the acetone to evaporate. A 2-mm-diameter disk of actively growing *L. terebrantis* (isolated LT23) mycelia on PDA was placed face down on the surface of the medium. The vials were capped and incubated at 24°C in the dark. Mean linear growth (millimeters) at three days was calculated. Means were compared using Duncan's multiple range test in the general linear models procedure in SAS (SAS Institute, 1982).

Nonpolar extracts could not be incorporated effectively into molten PDA. Therefore, mycelia of *L. terebrantis* and *L. procerum* were expoed to atmospheres saturated with pure synthetic monoterpenes found to be most prevalent in red pine—α-pinene, camphene, β-pinene, 3-carene, myrcene, limonene, and γ-terpinene (Klepzig et al., 1995b; Raffa and Smalley, 1995). Each assay chamber consisted of a sterile 90- × 15-mm plastic Petri plate (Fisher Scientific, Pittsburgh, Pennsylvania) containing PDA. Each monoterpene (200 μl) was dispensed directly onto a filter paper disk (0.45 cm diameter) fastened to the plate lid with paraffin wax. A disk (0.5 cm diameter) of either *L. terebrantis* (isolate K9013) or *L. procerum* (isolate LP5) mycelia on PDA was placed face down in the center of the assay plate. The lid was sealed into place with Parafilm, and assay chambers were incubated inverted at 20°C in darkness. Chambers with sterile filter paper fastened to their lids served as controls. This experiment was replicated 10 times for each fungus–monoterpene combination (control and myrcene treatments were replicated 20 times). Mean colony diameters at five days were calculated and compared using the protected least squares means procedure in ANOVA (Abacus Concepts, 1989).

Effect of Root Disease on Bark Beetle Host Selection Behavior

We extracted phloem tissue from mature, field-grown red pine to examine the effects of root infection with *Leptographium* on defense against insects and fungi. Test trees were in a 25-year-old red pine plantation in Sauk County, Wisconsin. We had previously determined the levels of infection with *Leptographium* spp. in these trees, relative to their distance from the decline epicenter, by examining and culturing from excavated roots (Klepzig et al., 1991). Inoculations, sampling, and chemical extractions were conducted as described above for trees estimated to have high and low levels of root infection, based on prior

excavations. Monoterpene extracts from constitutive and reaction tissue from these trees, as well as synthetic monoterpenes at concentrations found within these tissues, were incorporated into medium and applied in two-way choice host selection assays with *H. porculus* and *I. pini* as described above.

RESULTS

Effects of Constitutive and Induced Allelochemistry on Host Selection Behavior of Subcortical Insects

Polar Extracts. The relative effects of polar extracts of constitutive versus induced-reaction tissue on beetle tunneling varied between species (Table 1A).

TABLE 1. EFFECT OF CONSTITUTIVE AND INDUCED RED PINE PHLOEM EXTRACTIVES ON HOST SELECTION BEHAVIORS BY *H. porculus* AND *I. pini*[a]

		Distance		Time	
Insect	Comparison	cm	*P*	%	*P*
A. Polar extracts					
D. valens	Constitutive	4.7 (0.8)	0.06	62.5 (9.5)	0.05
	Inoculated	2.3 (0.9)		35.0 (10.3)	
H. porculus	Constitutive	2.2 (0.6)	ns	34.0 (6.6)	0.07
	Inoculated	1.3 (0.3)		19.0 (4.9)	
I. pini	Constitutive	0.7 (0.2)	ns	20.0 (4.2)	ns
	Inoculated	0.6 (0.1)		17.5 (4.0)	
B. Nonpolar extracts					
H. porculus	Constitutive	6.9 (1.0)	0.0001	74.2 (7.1)	0.0001
	Inoculated	1.1 (0.4)		15.5 (6.0)	
	Constitutive	4.5 (0.8)	ns	49.0 (8.3)	ns
	Mechanical Wound	2.9 (0.7)		34.0 (7.9)	
	Mechanical Wound	5.1 (0.9)	ns	50.0 (8.2)	ns
	Inoculated*	4.0 (1.2)		31.0 (8.2)	
I. pini	Constitutive	7.0 (1.1)	ns	63.0 (8.1)	0.0002
	Inoculated	4.4 (1.4)		20.0 (6.4)	
	Constitutive	3.1 (0.9)	ns	29.0 (5.5)	ns
	Mechanical Wound	3.7 (0.7)		37.0 (7.0)	
	Mechanical Wound	8.5 (1.7)	.01	71.5 (5.7)	0.0001
	Inoculated*	3.3 (1.1)		14.7 (5.0)	

[a]Media amended with extracts from mature red pine trees that were left unwounded (constitutive), mechanically wounded, or wound-inoculated with *L. terebrantis* and sampled three weeks after inoculation, except*, sampled one week after inoculation. Mean (standard error) tunneling and percent of assay period spent on each side in two-way choice assays. Duration of assays = 5 days. $N = 20$ for all comparisons.

D. valens tunneled further and remained for longer periods in extracts of constitutive than reaction tissue. The distances tunneled by *H. porculus* in medium containing extracts of constitutive and reaction tissue did not significantly differ. However, they remained on the side of the assay chamber containing constitutive extracts for a longer period. *Ips pini* showed no significant preferences between polar extracts of constitutive and reaction tissue.

Nonpolar Extracts. Host condition significantly affected tunneling by *H. porculus* and *I. pini* in nonpolar extracts from red pine. In two-way choice assays, *H. porculus* tunneled over six times as far, and spent nearly five times as much time, in extracts from constitutive than reaction tissue (Table 1B). These beetles exhibited no significant preference between extracts from constitutive and mechanically wounded tissue.

In choice assays involving nonpolar extracts of constitutive and reaction tissue, *I. pini* exhibited preferences similar to those of *H. porculus* (Table 1B). *Ips pini* spent over three times as much time within the side of the assay chamber containing extracts from constitutive tissue than in extracts from reaction tissue. However, the distances tunneled by beetles in these extracts did not significantly differ. *Ips pini* did not exhibit clear preferences between extracts from constitutive versus mechanically wounded tissue.

Synthetic Monoterpenes. Synthetic monoterpenes significantly affected *I. pini* behavior. *Ips pini* tunneled nearly three times more, and spent over four times more time, within the side of the assay chamber containing medium amended with the concentration of α-pinene found in constitutive tissue than in reaction tissue (Table 2). *Ips pini* also tunneled over five times farther, and spent over twice as much time within medium amended with the concentration of α-pinene found in mechanically wounded tissue than in the concentration found in reaction tissue. However, *I. pini* did not exhibit significant preferences between α-pinene concentrations found in constitutive versus mechanically wounded tissue.

In two-way choice assays, *I. pini* exhibited no significant preference between medium amended with the concentrations of β-pinene found in constitutive versus reaction tissue or between concentrations found in mechanically wounded versus reaction tissue (Table 2). *Ips pini* did tunnel over twice as much within medium containing the concentrations of β-pinene found in mechanically wounded as in concentrations found in constitutive tissue.

In no-choice assays, monoterpene concentration significantly affected beetle behavior. *Ips pini* tunneled over five times more in medium containing the lowest concentration of α-pinene tested (3.4 mg/g, only slightly lower than the concentration found in constitutive red pine phloem) than in medium containing the highest concentration of α-pinene tested (343.2 mg/g, only slightly higher than the concentration found in wound inoculated red pine phloem) (Figure 1A). A linear equation described the effects of α-pinene on *I. pini* feeding: $y = -0.004x$

TABLE 2. EFFECTS OF SYNTHETIC MONOTERPENES ON HOST SELECTION BEHAVIOR BY *I. pini*[a]

Extracts simulated using synthetic monoterpenes	Comparison	*N*	Distance		Time	
			cm	*P*	%	*P*
α-Pinene	Constitutive	10	1.6 (0.4)	0.05	46.0 (9.9)	0.003
	Inoculated		0.6 (0.2)		10.0 (4.4)	
	Constitutive	10	4.3 (0.9)	ns	30.0 (6.8)	ns
	Mechanical wound		3.3 (1.2)		22.0 (5.5)	
	Mechanical wound	19	5.9 (1.6)	0.009	57.8 (9.5)	0.008
	Inoculated		1.1 (0.4)		22.3 (8.5)	
β-Pinene	Constitutive	10	2.3 (0.8)	ns	12.0 (5.3)	ns
	Inoculated		0.7 (0.5)		4.0 (4.0)	
	Constitutive	10	3.8 (1.4)	0.0008	22.0 (5.5)	ns
	Mechanical wound		10.2 (1.5)		34.0 (5.2)	
	Mechanical wound	10	1.9 (0.5)	ns	10.0 (3.3)	ns
	Inoculated		4.8 (2.2)		26.0 (9.9)	
All	Constitutive	20	8.2 (1.8)	ns	40.0 (5.6)	ns
	Inoculated		5.2 (1.6)		35.0 (10.3)	

[a] Mean (standard error) tunneling and percent of assay period spent on each side in two-way choice assays. Media amended with pure monoterpenes at concentrations similar to those found in nonpolar extracts of mature red pine trees which were left unwounded (constitutive), mechanically wounded, or, wound-inoculated with *L. terebrantis* and sampled three weeks after inoculation. Duration of assay = 4 days. All = α-pinene, camphene, β-pinene, 3-carene, myrcene, and limonene combined.

+ 1.717; $r^2 = 0.974$; where y is the distance tunneled by the beetle, and x is the α-pinene concentration in the media. Likewise, *I. pini* tunneled over eight times more in medium containing the lowest concentration of β-pinene tested (3.4 mg/g, over 30 times higher than the concentration found in constitutive red pine phloem) than in medium containing the highest concentration of β-pinene tested (343.2 mg/g, nearly three times higher than the concentration found in wound inoculated red pine phloem) (Figure 1B). A linear equation described the effects of β-pinene on *I. pini* feeding: $y = -0.004z + 1.323$; $r^2 = 0.903$; where y is the distance tunneled by the beetle, and z is the β-pinene concentration in the media.

Effects of Induced and Constitutive Allelochemistry on Fungal Germination and Growth

Germination of both *L. terebrantis* and *L. procerum* was reduced by increasing concentrations of both α-pinene and β-pinene (Figure 2). At most doses tested, β-pinene was more inhibitory to both fungi than α-pinene. The

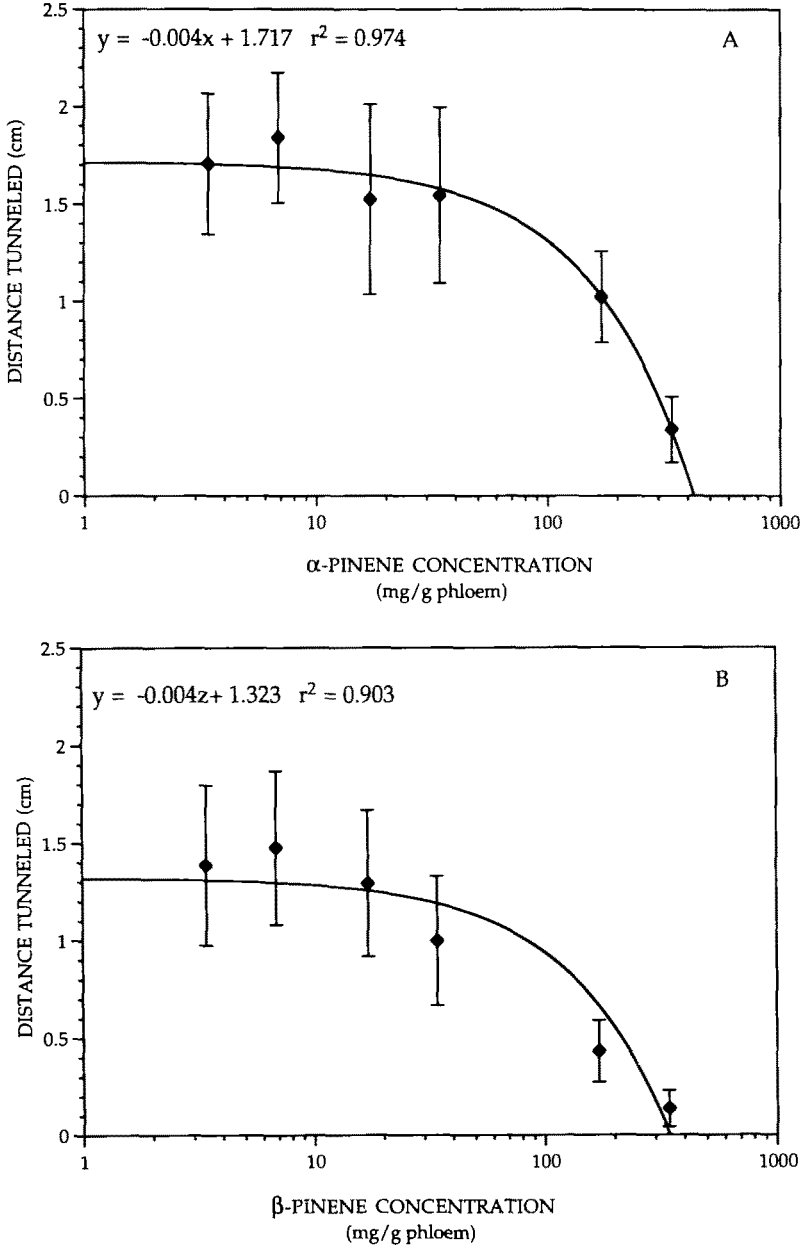

FIG. 1. Effects of synthetic monoterpenes on *I. pini* tunneling (mean ± SE). No-choice assay for two days in phloem-based medium amended with (A) α-pinene, $df = 20$, F 2.93, $P < 0.0001$, or (B) β-pinene, $df = 10$, $F = 2.15$, $P < 0.02$.

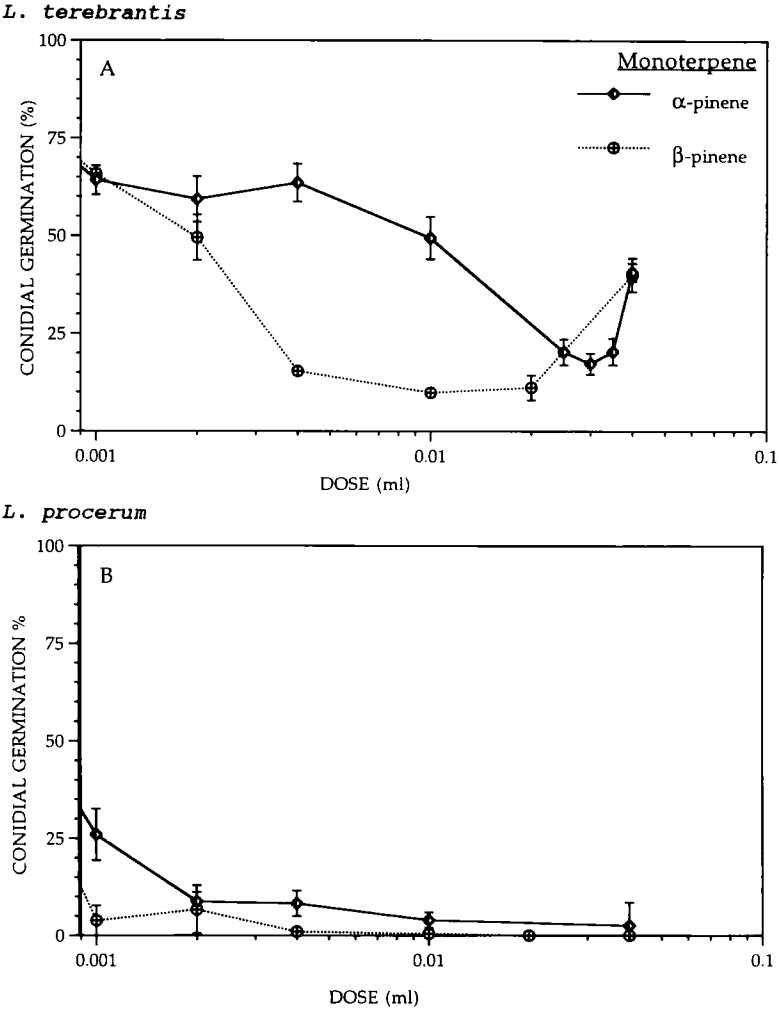

FIG. 2. Effects of α-pinene and β-pinene on germination (mean ± SE) of *L. terebrantis* and *L. procerum* conidia. Fungus by monoterpene interaction: $df = 24$, $F = 93.9$, $P < 0.0001$. Mean percent germination (and standard error) of fungi not exposed to monoterpenes: *L. terebrantis* = 98.0 (0.6) and *L. procerum* = 89.9 (2.3).

highest concentrations of both α-pinene and β-pinene, however, resulted in less inhibition of *L. terebrantis* germination than did intermediate concentrations. No such reduction in inhibitory activity at high concentrations was observed with *L. procerum*.

Polar Extracts. Polar extracts from both constitutive and reaction tissue reduced fungal growth (Figure 3). Extracts from constitutive tissue inhibited mycelial growth of *L. terebrantis* by approximately 15% relative to the control. Extracts of reaction tissue that had been mechanically wounded, inoculated with killed *L. terebrantis*, or inoculated with living *L. terebrantis* inhibited growth equally. Growth was approximately 44% lower on media containing these extracts than on media containing no additives. Medium amended with acetone alone did not differ in inhibitory activity from unamended medium.

Synthetic Monoterpenes. Mycelial growth of *L. terebrantis* was significantly inhibited by vapors of pure α-pinene, camphene, β-pinene, limonene,

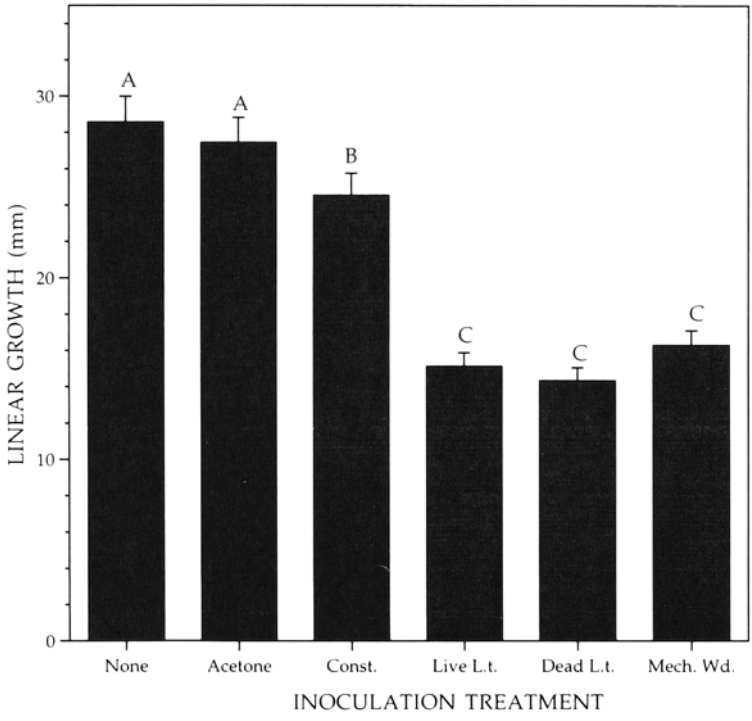

FIG. 3. Effect of acetone–methanol extracts on linear growth (mean ± SE) of *L. terebrantis*. Means followed by different letters are significantly different at $P < 0.05$ by Duncan's multiple range test. Controls: potato dextrose agar (PDA) only (None); PDA + acetone (Acetone). Sources of extracts: constitutive stem tissue (Const.); stem tissue one week after inoculation with viable *L. terebrantis* (Live L.t.); stem tissue 1 week after inoculation with nonviable *L. terebrantis* (Dead L.t.) stem tissue one week after mechanical wounding (Mech. Wd.). $df = 5$, $F = 63.79$, $P < 0.0001$.

TABLE 3. EFFECTS OF PURE MONOTERPENES ON GROWTH AND GERMINATION OF FUNGI[a]

	Mean linear growth (cm)		Mean conidial germination (%)	
Monoterpene	*L. terebrantis*	*L. procerum*	*L. terebrantis*	*L. procerum*
α-Pinene	2.71 (0.04) d	1.25 (0.02) b	40.7 (2.3) e	27.9 (3.0) e
Camphene	2.20 (0.00) e	0.02 (0.01) c	45.9 (2.6) ce	46.6 (3.5) d
β-Pinene	2.61 (0.05) d	1.19 (0.01) b	58.8 (2.5) bd	25.3 (5.2) e
3-Carene	3.34 (0.02) ab	1.35 (0.02) ab	52.7 (2.3) bc	49.7 (4.4) d
Myrcene	3.36 (0.07) ab	1.29 (0.04) ab	60.7 (3.0) ad	68.2 (2.7) c
Limonene	3.26 (0.10) c	1.24 (0.06) ab	76.2 (2.2) a	74.9 (2.5) b
γ-Terpinene	1.60 (0.03) f	0.96 (0.99) c	68.4 (3.2) a	23.2 (2.1) e
Control	3.52 (0.16) a	1.33 (0.02) a	98.0 (0.6) a	89.8 (2.3) a

[a]Mean (standard error) linear growth (cm) of mycelia and mean (standard error) germination (%) of conidia exposed to saturated atmospheres of pure monoterpenes found in red pine reaction tissue and to no monoterpenes (control). Linear growth measured at five day's exposure. Percent germination measured at 1 day of exposure. Means within a column followed by different letters are significantly different at $P < 0.05$ as determined by protected least squares means procedure.

and γ-terpinene (Table 3). Growth of *L. procerum* was significantly inhibited by α-pinene, camphene, β-pinene, and γ-terpinene. The greatest inhibition against both fungi was exerted by camphene and γ-terpinene, which are only trace components in red pine. Among the major components of host monoterpenes, α-pinene and β-pinene were the most inhibitory.

Saturated atmospheres of the predominant monoterpenes in red pine also reduced spore germination of both *L. terebrantis* and *L. procerum* (Table 3). All monoterpenes significantly inhibited germination of *L. procerum* relative to the control. Germination by *L. terebrantis* was significantly inhibited by α-pinene, camphene, β-pinene, and 3-carene. α-Pinene and camphene were the most potent inhibitors of *L. terebrantis*, inhibiting germination by 59% and 53%, respectively. γ-Terpinene, α-pinene, and β-pinene were the most potent inhibitors of *L. procerum*, inhibiting germination by 74%, 69%, and 72%, respectively.

Effect of Root Disease on Bark Beetle Host Selection Behavior

Nonpolar extracts from stem phloem of trees with high and low levels of root infection differentially affected the behavior of *I. pini* and *H. porculus*. In two-way choice assays, *H. porculus* showed no significant preferences between nonpolar extracts from healthy versus root diseased trees (Table 4A). This was true regardless of whether the extracts came from constitutive or reaction tissue.

Ips pini tunneled over four times more, and spent nearly three times more

TABLE 4. EFFECT OF NONPOLAR PHLOEM EXTRACTS AND SYNTHETIC MONOTERPENES FROM HEALTHY AND ROOT-DISEASED RED PINES ON HOST SELECTION BEHAVIORS BY *H. porculus* and *I. pini*[a]

	Comparison		Distance		Time	
Insect	Tree condition	Tree tissue	cm	*P*	%	*P*
A. Nonpolar extracts						
H. porculus	Healthy	Constitutive	6.3 (2.1)	ns	53.3 (14.9)	ns
	Diseased	Constitutive	4.7 (1.7)		46.6 (14.9)	
	Healthy	Inoculated	4.2 (1.9)	ns	50.0 (13.4)	ns
	Diseased	Inoculated	2.4 (0.8)		46.0 (14.6)	
I. pini	Healthy	Constitutive	4.8 (1.2)	0.01	52.0 (13.0)	0.05
	Diseased	Constitutive	1.1 (0.6)		18.0 (9.6)	
	Healthy	Inoculated	2.4 (1.0)	0.06	16.0 (8.8)	0.01
	Diseased	Inoculated	6.0 (1.5)		56.0 (11.0)	
B. Synthetic monoterpenes						
I. pini	Healthy	Inoculated	1.0 (0.6)	ns	7.5 (3.6)	ns (0.13)
	Diseased	Inoculated	2.5 (1.3)		22.5 (8.8)	

[a]Mean (standard error) tunneling and percent of assay period spent on each side in two-way choice assay. A: Nonpolar extracts of healthy vs. root-diseased mature red pine trees that were unwounded (constitutive) or wound-inoculated with *L. terebrantis* and sampled three weeks after inoculation; B: Media amended with concentrations of pure monoterpenes similar to concentrations found in extracts of healthy and root-diseased mature red pine trees that were wound inoculated with *L. terebrantis* and sampled three weeks after inoculation. Duration of assay = 3 days. $N = 20$ for all comparisons.

time, within extracts of constitutive tissue from healthy trees than in extracts from trees with high levels of root infection. However, this pattern was reversed when tree defensive capacity was challenged by wound inoculation with *L. terebrantis*. *Ips pini* tunneled over twice as much, and spent over three times more time, within extracts of reaction tissue from trees with high than low levels of root infection (Table 4A).

Synthetic pure monterpenes incorporated into media at concentrations equivalent to those found in reaction tissue from trees with high and low levels of root infection did not significantly affect *I. pini* behavior (Table 4B).

DISCUSSION

These experiments demonstrate the ability of allelochemically based defenses in red pine to affect both insect and fungal parasites. In this system, the early stages of combined parasitic invasion can apparently be interrupted by host

chemical defenses. Beetles and fungi invading red pine are only slightly (or not at all) inhibited by the levels of polar compounds (phenolics) and nonpolar compounds (monoterpenes) present in the constitutive tissue that they first encounter. However, as the beetles tunnel into subcortical tissue, they elicit an induced response from the host (Cheniclet et al., 1988; Miller et al., 1986; Berryman, 1988; Raffa and Smalley, 1995). The resulting elevated concentrations of phenolics and monoterpenes can significantly inhibit the germination of fungal spores or inhibit subsequent hyphal development. Continued insect tunneling and fungal development elicit further host reactions, which are usually sufficient to repel the attack in vigorous, healthy trees.

The effects of particular components of the induced host response vary with the type and species of invading parasite. In this system, phenolics appear to affect fungi to a greater degree than insects but are only moderately inhibitory to both. Conversely, monoterpenes in red pine are highly active in inhibition of both insects and fungi. Based on the results with synthetic monoterpenes, these compounds can partially, but not fully, explain beetle preferences for various host extracts.

H. porculus and *I. pini* both tended to prefer constitutive to induced reaction tissue, at least as indicated by beetle location. However, only *I. pini* discriminated between reaction tissues elicited in response to mechanical wounds versus fungal inoculations. Likewise, only *I. pini* discriminated between extracts from healthy versus diseased trees. These results suggest that *I. pini* is more discriminating than *H. porculus* with regard to monoterpene concentrations and that responses to induced reactions are an essential component of *I. pini*'s overall host selection process. These preferences may relate to host selection behavior by *I. pini* in the field. That is, *I. pini* males must discriminate between vigorous hosts capable of mounting an effective defense and stressed hosts that might be more suitable for colonization and subsequent brood production. *I. pini* preferred extracts from constitutive tissue from healthy trees to extracts from similar tissue from diseased trees. However, monoterpene concentrations were very low in constitutive tissues (Klepzig et al., 1995b), and this may merely represent a stimulatory effect of low concentrations of monoterpenes on *I. pini* tunneling. In reaction tissue resulting from wound inoculation with *L. terebrantis*, this preference was reversed. Soon after *I. pini* enters red pine, it inoculates its associated fungus, *O. ips*, into the phloem and initiates a rapid induced response (Raffa and Smalley, 1988). This is the type of tissue with which *I. pini* is most likely confronted as it begins the process of gallery construction that may lead to pheromone production and subsequent mass attack by other *I. pini*. Thus, the ability of a tree to mount an effective induced response may be as important, or more important, than its constitutive allelochemical composition. We currently know very little about the biology and behavior of *H. porculus*, so results with this root-feeding species are more difficult to interpret (Klepzig, 1994).

The closely related fungi studied here also differed in their sensitivities to host extracts. The germination and growth of *L. procerum* were strongly inhibited by saturated atmospheres of monoterpenes. The germination and growth of *L. terebrantis* were also inhibited by monoterpenes, but to a lesser extent. The biological significance, if any, of the decreased levels of inhibition of germination of *L. terebrantis* conidia by the highest concentrations of α- and β-pinene is unclear. These higher doses may be artificially high and/or the decreased level of inhibition at these doses may be an artifact of the assay chamber design. The differences between the two fungi in allelochemical tolerance, however, may relate to life history traits of these two fungi. *L. procerum* is only weakly virulent in red pine and most other conifers. *L. terebrantis*, the more allelochemically tolerant species, may be among the most virulent of the bark beetle-associated fungi (Harrington, 1993). In two growth-chamber studies *L. terebrantis*, but not *L. procerum*, was able to kill red pine seedlings (Klepzig, 1994). Although this pattern does not demonstrate a definite link between allelochemical tolerance and virulence in these plant parasites, it is consistent with this hypothesis and argues for further work on this aspect of conifer–scolytid–fungal interactions.

Biotic and abiotic stresses can impair the defense mechanisms described above. Trees whose roots have been extensively colonized by parasitic organisms are less able to produce reaction tissue that is inhibitory to *I. pini*. These behavioral reactions correspond to reduced levels of induced allelochemical accumulation, following *I. pini–O. ips* attack in root-infected trees (Klepzig et al., 1995b). Thus, successful colonization by the root-colonizing beetle *H. porculus* and its associated fungus *L. terebrantis* appears to predispose red pine to subsequent attack and colonization by the main stem-colonizing beetle *I. pini* and its associated fungus *O. ips*. Tree mortality data are consistent with this proposed mechanism (Klepzig et al., 1991; Raffa and Klepzig, 1996). Abiotically stressed trees can also be more susceptible to infection by these facultatively pathogenic fungi (Klepzig, 1994). As with most infection, the stem subcortical tissue of light-limited trees showed altered patterns of allelochemical induction in response to simulated attack (Klepzig et al., 1995b). Both of these examples of increased susceptibility in relation to biotic and abiotic stress can be at least partially explained by changes in the carbon-based defensive chemistry. That is, the observed chemical changes are consistent with physiological changes associated with impaired host abilities to compete for resources. For example, diseased roots and shaded needles are less able to capture water and nutrients, and sunlight, respectively.

The relationships among trees physiology and plant parasites described here have implications for the ecology and management of forest ecosystems. In particular, they suggest a probable sequence for the multiple species interactions involved in a decline disease affecting red pine (Klepzig et al., 1991). The most

frequent and most severe examples of this decline typically occur in trees growing off site, often on poor soils, and under potentially resource-limiting conditions. The resulting chronic stresses may represent predisposing factors as defined by Manion (1991). Likewise, some short-term stresses that can serve as inciting factors in this decline have been identified. These include reduced light availability, root disease, lightning strike, and/or root damage due to temperature or moisture extremes. However, the exact identity, relative incidence, and overall importance of various inciting factors in red pine decline disease remain unclear. Contributing factors appear to include a complex of subcortical root- and stem-infesting insects and their associated fungi partially described here (Klepzig et al., 1991). Induced defensive reactions to attack by this multispecies complex appear to be compromised by both the predisposing and contributing factors. This may allow increasingly successful attacks by root insects and fatal attacks by *I. pini–O. ips* complexes (Klepzig et al., 1991). This hypothetical framework has also led to suggested strategies for managing red pine decline disease, including severing root grafts in advance of the spread of the root-infecting fungi and planting trees in mixed-species blocks.

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OPTIMIZATION OF SEMIOCHEMICAL-BASED TRAPPING OF *Metamasius hemipterus sericeus* (OLIVIER) (COLEOPTERA: CURCULIONIDAE)

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Abstract—Response of adults of the West Indian sugarcane weevil, *Metamasius hemipterus sericeus*, to various semiochemical treatments and physical trap designs was studied in southern Florida in field-grown banana and Canary Island date palms. Ethyl acetate released alone at 860–1007 mg/day was as effective for the capture of *M. h. sericeus* as a combination of ethyl acetate (844–919 mg/day), ethyl propionate (348–362 mg/day), and ethyl butyrate (117–137 mg/day) and in one trial was more effective than fermenting sugarcane (250 g), ethyl propionate alone (353–384 mg/day), ethyl butyrate alone (123–174 mg/day), or no treatment. Ethyl acetate released alone at 675–683 mg/day was as attractive as 250 g of fermenting sugarcane or the racemic blend of the male-produced aggregation pheromones [(±)-5-methyl-nonan-4-ol and (±)-2-methyl-heptan-4-ol (8:1 ratio) "metalure"] at 3 mg/day. Weevil counts increased with binary combinations of ethyl acetate, sugarcane, and/or metalure over these treatments alone and the ternary combination was two to three times more effective than any of the binary treatments. Attraction to ethyl acetate released alone at 777 mg/day with metalure was greater than to the hydrolysis products of ethyl acetate (ethanol and/or acetic acid each released at about 6–8 mg/day) with metalure. Weevil counts from traps baited with 250 g of sugarcane and metalure increased with increasing dose of ethyl

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acetate to about 400 mg/day and then appeared to plateau. Ethyl acetate (about 700 mg/day) and metalure increased weevil counts in traps with increasing amounts of sugarcane (0–2 kg). Molasses (45 g) + water (158 ml) substitutes for sugarcane were about as effective for capturing *M. h. sericeus* as 250 g of fermenting sugarcane [all treatments with ethyl acetate (847 mg/day) and metalure]. Early experiments used a lethal pitfall trap. We demonstrated that several alternative trap designs were more effective than the lethal pitfall trap for capturing weevils. Color and height (on ground vs. 1 m on pole) did not affect trap efficacy when baited with the ternary combination of ethyl acetate (672–825 mg/day), sugarcane (250 g), and metalure.

Key Words—Aggregation pheromones, attraction, banana, Canary Island date palm, ethyl acetate, kairomones, monitoring, *Phoenix canariensis*, West Indian sugarcane weevil, sugarcane, trapping.

INTRODUCTION

The West Indian sugarcane weevil, *Metamasius hemipterus sericeus* (Olivier), is an important pest of palms, sugarcane, pineapple, and bananas in the West Indies, Mexico, and Central and South America (Giblin-Davis et al., 1994b; Vaurie, 1966). Adult females lay eggs in cracks or damaged areas of the host plant or in the petioles or crown shaft of certain species of healthy palms. The larvae bore and feed in the host tissue, causing extensive physical damage that can lead to the death of the host (Giblin-Davis et al., 1994b). This weevil first became established in Dade County, Florida in about 1984 (Woodruff and Baranowski, 1985) and has recently become established in Broward and Palm Beach counties (Giblin-Davis et al., 1994b).

The container and field-grown ornamental palm industry in Florida is valued at more than $50 million per year. Current Florida Department of Plant Industry regulations stipulate that palm nurseries with *M. h. sericeus* must be quarantined and sprayed with insecticides until the weevils are no longer detectable in plants for sale or distribution. There is an urgent need for an effective, optimized, semiochemical-based lethal trap for monitoring and mass-trapping of *M. h. sericeus* in Florida.

Male-produced aggregation pheromones (4-methyl-nonan-5-ol and 2-methyl-heptan-4-ol) have been identified for *M. h. sericeus* (Perez et al., unpublished data; Rochat et al., 1993). Furthermore, early fermentation products from sugarcane and other plant tissues are very attractive to *M. h. sericeus* (Giblin-Davis et al., 1994b). Several kairomones (ethyl acetate, ethyl butyrate, and ethyl propionate) have been identified for *M. h. sericeus* from fermenting host tissue (Perez et al., unpublished data). We previously developed a lethal pitfall trap that can be baited with sugarcane or other fermenting fruit or palm tissues (Giblin-Davis et al., 1994b) and with identified kairomones or male-

produced aggregation pheromones. The purpose of this study was to further optimize the bait, design, height of placement, and color for a safe, lethal, semiochemical-based trap.

METHODS AND MATERIALS

Field Attraction to Synthetic Host Kairomones Compared with Sugarcane

Trap. A semiochemical-based lethal pitfall trap for *M. h. sericeus* (Giblin-Davis et al., 1994b) was used for field experiments comparing the attraction of the kairomones, ethyl acetate, ethyl propionate, or ethyl butyrate versus chopped sugarcane, *Saccharum officinarum* L. (250 g of 2- to 5-cm-long stem pieces). Each trap consisted of two translucent white, high-density polyethylene (HDPE) plastic tapered containers (Fisher Scientific Co., Pittsburgh, Pennsylvania), one 4.9 liter and the other 0.9 liter in size. Soapy water in the bottom of the larger container acted as the lethal agent. Two drainage holes in the sides, 6 cm from the bottom, allowed for rain overflow. The smaller container had a screened lid (8.5-cm screened opening), to retain the sugarcane and prevent weevils from interacting with it when inverted, and was suspended with a vinyl-coated wire inside the larger container. Each assembled trap was buried to within about 2.5 cm of the trap opening in the shade of a mature banana plant.

Kairomone Release. Two devices were used for the release of synthesized host kairomones: (1) 1 ml of a test chemical in a 2-ml Gold Brand ampule (Wheaton, Millville, New Jersey) (designated AMP) with a 4-mm-diam. opening, and (2) 4 or 8 ml of test chemical in a 14.8-ml glass vial (OD × H = 21 × 70 mm) without a top (11.4-mm-diam. opening) (4NT) (Giblin-Davis et al., 1994a). Release devices were placed in a 120-ml HDPE specimen container (Fisher Scientific) suspended from inside of the 0.9 liter test stimulus container by wires. The amount of each test semiochemical released was gravimetrically quantified at the end of each trapping period, and a mean daily release rate was calculated. The actual release rates would have varied during the day with changing temperature and relative humidity.

Low-Release Rate Experiment. Ethyl acetate (Fisher Scientific), ethyl propionate (Aldrich Chemical Co., Milwaukee, Wisconsin), or ethyl butyrate (Kodak Chemical Co., Rochester, New York), all ≥99% pure, were released individually using the AMP device and compared with a ternary combination of individually released chemicals, ethyl acetate released alone using the 4NT device, and 250 g of sugarcane. Both of the following experiments were conducted in a 15.5-ha, 2-year-old banana plantation, located near Homestead, Dade County, Florida. Two five-replicate experiments were done during February 3–9 and February 10–16, 1993. Treatments were rerandomized after the first trial.

Experimental Design and Analysis. All of the following experiments were designed and analyzed as follows, unless otherwise stated. Treatments were about 20 m apart in a randomized complete block design separated by ≥20 m. Total numbers of adult male and female *M. h. sericeus* collected per trap for each trapping period were used for analysis. Counts were $(x + 0.5)^{0.5}$ transformed and the proportion of females (female/female + male) were arcsine-transformed to make the variances independent of the means and a Statistical Analysis System general linear models procedure (SAS Institute, 1985) for unbalanced analysis of variance (ANOVA) was used. Untransformed means are presented. Significant differences ($P \leq 0.05$) among treatment means were separated using the least-square-difference (LSD) method (SAS Institute, 1985).

High-Release Rate Experiment. Ethyl acetate, ethyl propionate, and ethyl butyrate were released individually using the 4NT device and compared with a ternary combination of all three chemicals released individually, and a treatment of 250 g of sugarcane. Two five-replicate experiments were done during February 17–22 and February 24–March 1, 1993. Treatments were rerandomized after the first trial.

Field Attraction to Ethyl Acetate or Ethyl Acetate Hydrolysis Products, Sugarcane or Sugarcane Substitutes, and/or Metalure

Trap. Experiments were discontinued in the banana plantation because the bananas were removed and replaced with another crop. All subsequent field testing was done in a 1-ha field nursery of 6-year-old Canary Island date palms, *Phoenix canariensis* Hortorum ex Chabaud, interplanted with 6-year-old Sabal palms, *Sabal palmetto* (Walter) in Broward County, Florida. The *P. canariensis* palms were heavily infested with *M. h. sericeus*. Prelminary testing with the lethal pitfall trap at this site was hampered due to a large population of raccoons that disturbed the traps. Studies with marked dead weevils suggested that the raccoons were eating them. Preliminary field tests showed that duct-taping the lethal pitfall trap to a green metal fencepost with the trap opening at a 1-m height, between palm trees, stopped tampering by raccoons and appeared to have no effect on trap efficacy. Subsequent field experiments used this trap design and placement, unless otherwise stated (Figure 1A).

Metalure and Kairomone Release. Racemic 4-methyl-nonan-5-ol and 2-methyl-heptan-4-ol were synthesized (>95% purity) and released in an 8:1 ratio (metalure) from hermetically sealed polyethylene envelopes (total release rate 3 mg/day at 25°C) for all experiments using metalure in this paper. Metalure was released from outside the inner bucket of the lethal bucket trap. Ethyl acetate (8 ml) was released from a 14.8-ml glass vial (OD × H = 21 × 70 mm) with a screw top with a 4-mm-diam. hole (4DH) that was placed in a wired 25.9-ml polystyrene vial that hung outside the inner bucket (Giblin-Davis et al., 1994a).

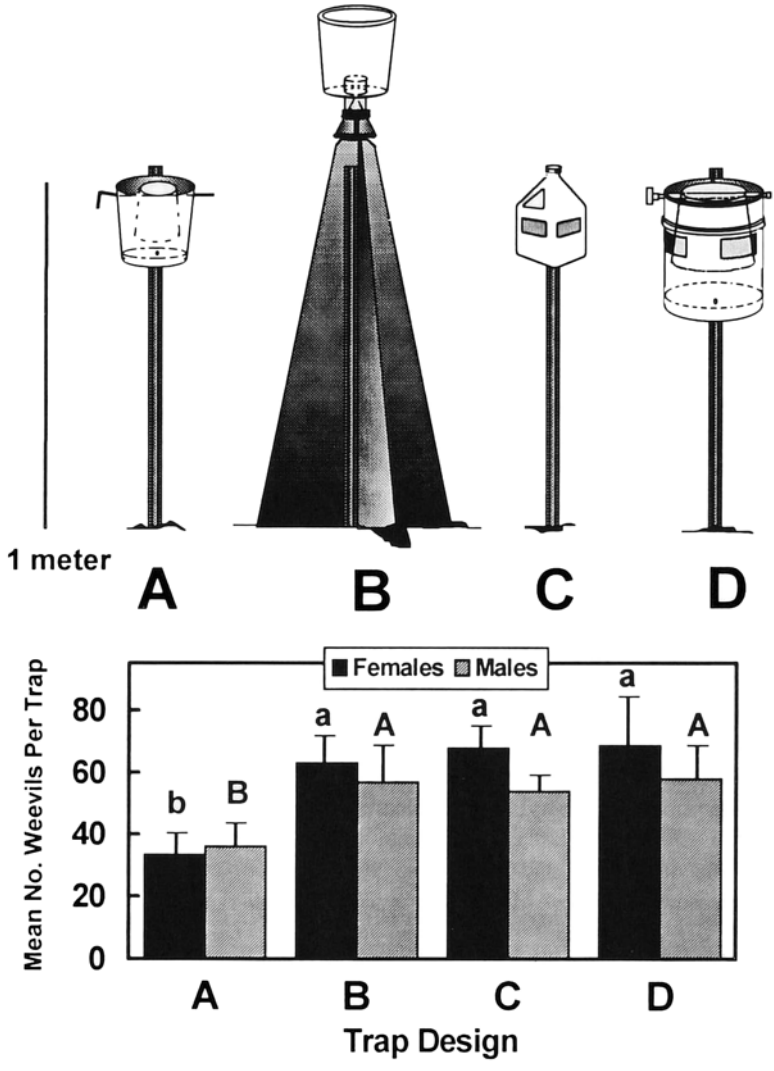

FIG. 1. Comparison of counts of *Metamasius hemipterus sericeus* females and males (mean ± SE) from different lethal trap designs in a Canary Island date palm planting in Broward County, Florida (March 3–7, 1995). Traps were baited with 250 g of sugarcane (treated to runoff with carbaryl at 1.11 ml a.i./liter of water), ethyl acetate (672 mg/day), and metalure [(±)-5-methyl-nonan-4-ol and (±)-2-methyl-heptan-4-ol, 8:1 ratio, total release rate of 3 mg/day] (7 replicates). (A) Lethal pitfall trap mounted on a pole; (B) modified Tedder's trap; (C) milk container trap on pole; and (D) 19-liter black bucket trap on pole shown with 4.9 liter stimulus container. Means followed by the same upper- or lowercase letter are not significantly different (least significant difference, $P \leq 0.05$). Count data were $(x + 0.5)^{0.5}$ transformed prior to analysis; untransformed means are presented.

Experiment 1: Individual, Binary, and Ternary Combinations. Two experiments, with treatments being replicated three and five times each during February 10–14 and February 14–18, 1995, respectively, were conducted to assess how ethyl acetate (released at 400–800 mg/day) would affect trap efficacy in all possible single, binary, and ternary combinations with 250 g of sugarcane and/or metalure. There were a total of seven treatments. The first three involved the release of metalure alone, 250 g of sugarcane alone, or ethyl acetate released alone from the 4DH device. The three possible binary combinations and one ternary combination of the above-mentioned individual treatments were also used. Treatments were rerandomized after the first trial.

Experiment 2: Field Attraction to Ethyl Acetate or its Hydrolysis Products. A five-replicate experiment was conducted during March 7–13, 1995, to determine if hydrolysis products of ethyl acetate (ethanol and/or acetic acid) were attractive to *M. h. sericeus* when released at rates similar to what is produced (6–8 mg/day) in ethyl acetate released from the 4DH release device (400–800 mg/day) (Oehlschlager et al., unpublished observation). Trap design was as previously described (Figure 1A). The five treatments were: no treatment, ethyl acetate released from the 4DH device, ethanol hermetically sealed in polyethylene envelopes and released at 6–8 mg/day at 25°C, acetic acid released at 6–8 mg/day at 25°C, and a combination of separately delivered ethanol and acetic acid (both released at 6–8 mg/day at 25°C). No sugarcane was used in this experiment, and all treatments received metalure.

Experiment 3: Ethyl Acetate Dose–Response with Sugarcane (250 g) and Metalure. A seven-replicate experiment was conducted during February 22–27, 1995, to determine how ethyl acetate release rates affected the efficacy of a trap baited with 250 g of sugarcane and metalure. The four treatments used were combinations of metalure and 250 g of sugarcane with no ethyl acetate, or ethyl acetate released from the AMP, 4DH, or 25 ml of ethyl acetate in a 40.7-ml glass vial (OD × H = 28 × 108 mm) without a top (17.8-mm-diam. opening) (11NT) (Giblin-Davis et al., 1994a). All buckets received a 120-ml HDPE specimen container for holding the ethyl acetate release devices, and it was suspended outside the inner bucket as previously described.

Experiment 4: Sugarcane Dose–Response with Ethyl Acetate (4DH device; 400–800 mg/day) and Metalure. A seven-replicate experiment was conducted during March 13–17, 1995, to determine how different amounts of sugarcane (0–250 g) affected the efficacy of a trap baited with ethyl acetate (4DH device) and metalure. The four treatments were combinations of metalure and ethyl acetate released from the 4DH device with no sugarcane, or 50, 100, or 250 g of sugarcane.

Another seven-replicate experiment was conducted during March 22–27, 1995, to determine if an increased amount of sugarcane (250 g, 500 g, or 2 kg) would affect trap efficacy of the ternary semiochemical combination (ethyl ace-

tate, sugarcane, and metalure). The trap design was modified to accommodate a larger amount of sugarcane (2 kg) as described in the trap optimization design section that follows (Figure 1D) except that a 4.9-liter HDPE container was used instead of the 0.9-liter container. The three treatments were combinations of metalure and ethyl acetate released from the 4DH device with 250 g, 500 g, or 2 kg of sugarcane.

Experiment 5: Sugarcane Substitutes. A five-replicate experiment was conducted during June 7–12, 1995, to evaluate molasses-based substitutes for sugarcane in black 19-liter bucket traps on green fenceposts as described (Figure 1D). All traps received metalure and ethyl acetate (4DH release device). The five treatments were no sugarcane, 250 g of sugarcane, a 36 × 43-cm cotton terry towel (Stout Industries) treated with 45 g of molasses (Grandma's "unsulphured" "mild flavor" Mott's U.S.A., Stamford, Connecticut), 45 g of molasses and 158 ml of tap water, or 45 g of molasses, 158 ml of tap water, and 0.15 g of Fleischman's active dry yeast (Specialty Brands, San Francisco, California).

Optimization of Trap Design, Placement, and Color Using Sugarcane (250 g), Ethyl Acetate (4DH Release Device), and Metalure as Bait

Design. Four trap designs were field-tested in a seven-replicate experiment during March 3–7, 1995, with metalure, ethyl acetate (4DH release device in the 29-ml polystyrene vial), and 250 g of sugarcane that was treated to runoff with Sevin liquid [21.3% carbaryl; 1-naphthyl *N*-methyl-carbamate (1.11 ml a.i./liter water)] (Figure 1A–D). The insecticide was used on all sugarcane to make the treatments semiochemically equal and to make designs 1B and 1C lethal to weevils that entered these traps. The designs follow: (A) A lethal pitfall trap (see above) that was duct-taped to a green metal fencepost that stood 1 m high (same as experiment 1). (B) A Tedder's (Tedder, unpublished data) modified boll weevil trap (Great Lakes IPM, Vestaburg, Michigan), that opened into a translucent white, 4.9-liter, HDPE stimulus container (where the sugarcane was placed) and was set on top of a 1.25-m-tall black triangular plastic base anchored to the soil with a green fencepost. The hole in the screen funnel of the boll weevil trap was expanded to a diameter of 8 mm and the metalure and the ethyl acetate were released from the outside of the stimulus container. (C) A clean 3.78-liter HDPE milk container, with three 4 × 10-cm openings cut 12 cm from the bottom, was attached to a green metal fencepost with duct tape. Pesticide-treated sugarcane was placed inside the container, and the metalure and ethyl acetate were hung from the outside. (D) A 19-liter black HDPE plastic bucket (United States Plastics Corp., Lima, Ohio) with three 4 × 13-cm openings spaced 10 cm apart and 21 cm from the bottom was attached to a green metal fence post with an elastic cord and duct tape. A translucent white,

0.9-liter, HDPE stimulus container with a fine screened lid was inverted and suspended 2 cm from the opening of the bucket with a PVC crossbar capped with a PVC cap and tee. Drainage holes were 5 cm from the bottom of the 19-liter bucket and a detergent solution was used to kill the weevils entering the trap (Giblin-Davis et al., 1994a).

Height. The lethal trap (see Figure 1D) was field-tested with metalure, ethyl acetate (4DH release device), and 250 g of sugarcane (no pesticide) on the ground versus mounted at 1-m height on a green metal fencepost. The ground-mounted trap was the same as the pole mounted trap except that it was anchored to the ground with metal posts driven through the PVC tees (see trap 1A of Giblin-Davis et al., 1994a). The experimental design and analysis were as previously described except that a *t* test was used for testing significance. The 10-replicate experiment was conducted during March 27–31, 1995.

Color. The ground-mounted 19-liter bucket trap (see Height section) baited with metalure, ethyl acetate (4DH release device), and 250 g of sugarcane (no pesticide) was used to assess the effects of bucket color on trap efficacy. There were two experiments. The first five-replicate experiment used black, white, and gray buckets (United States Plastics) and was conducted during April 21–25, 1995. The second five-replicate experiment used black, red, green, yellow, and blue buckets (United States Plastics) and was conducted during May 8–11, 1995.

RESULTS AND DISCUSSION

Field Attraction to Synthetic Host Kairomones Compared with Sugarcane

Low-Release Rate Experiment. There were no significant differences between any of the treatments using the lethal pitfall trap in the banana field for the capture of *M. h. sericeus* in either time replicate of this experiment (F = 1.09 and 1.87; df = 6, 4 and 6, 4; P = 0.3980 and 0.1281 for time replicates 1 and 2, respectively).

High-Release Rate Experiment. Ethyl acetate released alone at (mean release rates for time replicate 1;2) 860;1007 mg/day from lethal pitfall traps in banana fields was as effective for the capture of *M. h. sericeus* as a combination of ethyl acetate (833;919 mg/day), ethyl propionate (348;362 mg/day), and ethyl butyrate (117;137 mg/day) and in time replicate 2 was more effective than fermenting sugarcane (250 g), ethyl propionate alone (353;384 mg/day), ethyl butyrate alone (123;174 mg/day), or no treatment (Figure 2). The proportion of females per trap was about 68–70% for the different treatments for both time replicates. In general, electrophysiologically active host kairomones (ethyl acetate and, to a lesser degree, ethyl propionate) were as attractive as fermenting sugarcane when released alone or in a ternary combination with ethyl butyrate

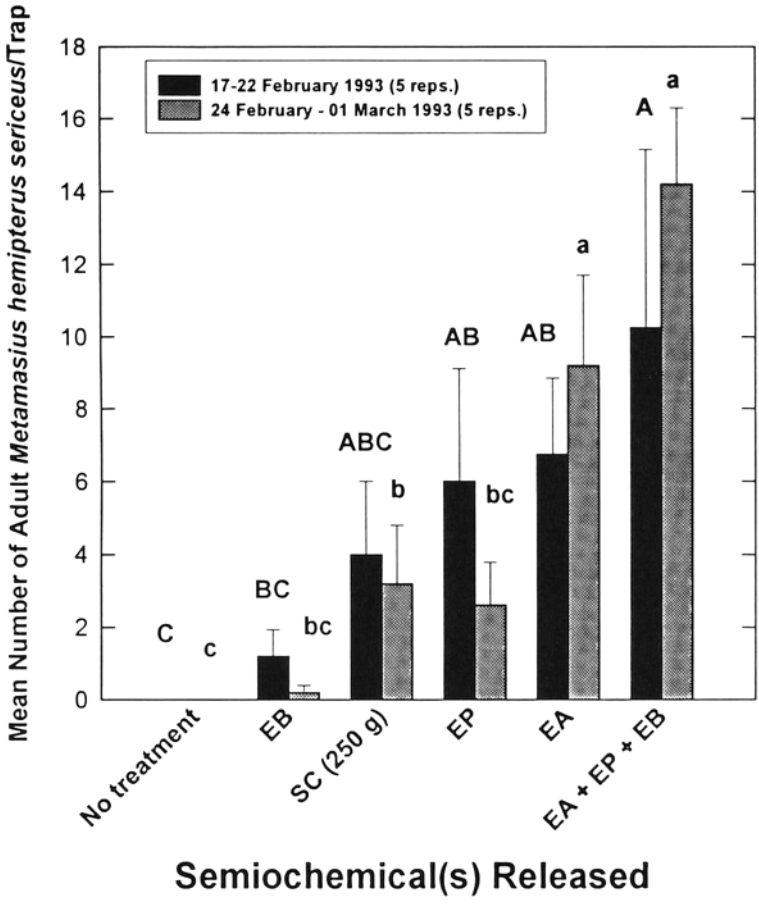

FIG. 2. Comparison of counts of *Metamasius hemipterus sericeus* adults (mean ± SE) from lethal pitfall traps in a banana planting in Dade County, Florida. Traps were baited with 250 g of sugarcane (SC); ethyl acetate alone (EA, mean release rates for time replicates 1;2) at 844;919 mg/day; ethyl propionate alone (EP) at 353;384 mg/day; ethyl butyrate alone (EB) at 123;174 mg/day; a ternary combination of EA, EP, and EB released individually at 860;1007, 348;362, and 117;137 mg/day; and no-treatment control. Means followed by the same upper- or lowercase letter are not significantly different (least significant difference, $P \leq 0.05$). Count data were $(x + 0.5)^{0.5}$ transformed prior to analysis; untransformed means are presented.

at the rates tested (Figure 2). One explanation for the lack of improvement in trap counts with the ternary treatment of ethyl acetate, ethyl propionate, and ethyl butyrate compared with the individual releases of these molecules (Figure 2) is that they may be stimulating the same receptors. The results from this experiment differ from what was observed for the palmetto weevil, *Rhynchophorus cruentatus* (F.), where ethyl acetate synergized attraction of the synthesized male-produced aggregation pheromone [(±)-5-methyl-octan-4-ol; cruentol] but was not attractive by itself at different doses (Giblin-Davis et al., 1994a). These results (Figure 2) prompted us to do experiments 1–5 to help optimize a semiochemical bait for *M. h. sericeus* in the field.

Field Attraction to Ethyl Acetate or Ethyl Acetate Hydrolysis Products, Sugarcane or Sugarcane Substitutes, and/or Metalure

Experiment 1: Individual, Binary, and Ternary Combinations. Ethyl acetate released alone from the 4DH device was as effective for capture of both sexes of *M. h. sericeus* in trap design A in the Canary Island date palm grove as 250 g of fermenting sugarcane, but more effective than metalure (F = 19.88 and 48.64; df = 6, 2 and 6, 4; P = 0.0001 and 0.0001 for time replicates 1 and 2, respectively). Weevil counts increased with all three binary combinations of ethyl acetate, sugarcane, and/or metalure compared with these semiochemical treatments alone. For males, binary combinations with ethyl acetate (ethyl acetate + metalure or ethyl acetate + sugarcane) were more effective than sugarcane + metalure (Figure 3). The ternary combination increased trap efficacy two to three times over any of the binary treatments for both time replicates (Figure 3). There was no difference in the release rates of ethyl acetate from the different treatments for either time replicate; mean release rates were 683 and 675 mg/day for time replicates 1 and 2, respectively (F = 3.28 and 0.75; df = 3, 2 and 3, 4; P = 0.1005 and 0.5446). There also was no difference in the proportion of females per trap for the different treatments for time replicate 1 (41%) (F = 2.50; df = 6, 2; P = 0.0892). There were differences in the proportion of females per trap in time replicate 2 (F = 10.37; df = 6, 4; P = 0.0001). Comparisons between time replicates 1 and 2 showed no consistent trends between the proportion of females and treatment suggesting that these differences may not be real.

These results are similar to the work of Jaffé et al. (1993), who reported that ethyl acetate (release rate undetermined) increased capture of *Rhynchophorus palmarum* (L.) in lethal and nonlethal traps baited with sugarcane and its major aggregation pheromone [2(*E*)-6-methyl-2-hepten-4-ol; rhynchophorol]. That sugarcane contributed to the attraction of ethyl acetate in binary or ternary combinations with metalure suggests that there are other kairomones to be identified from fermenting sugarcane. Research with different species of palm and

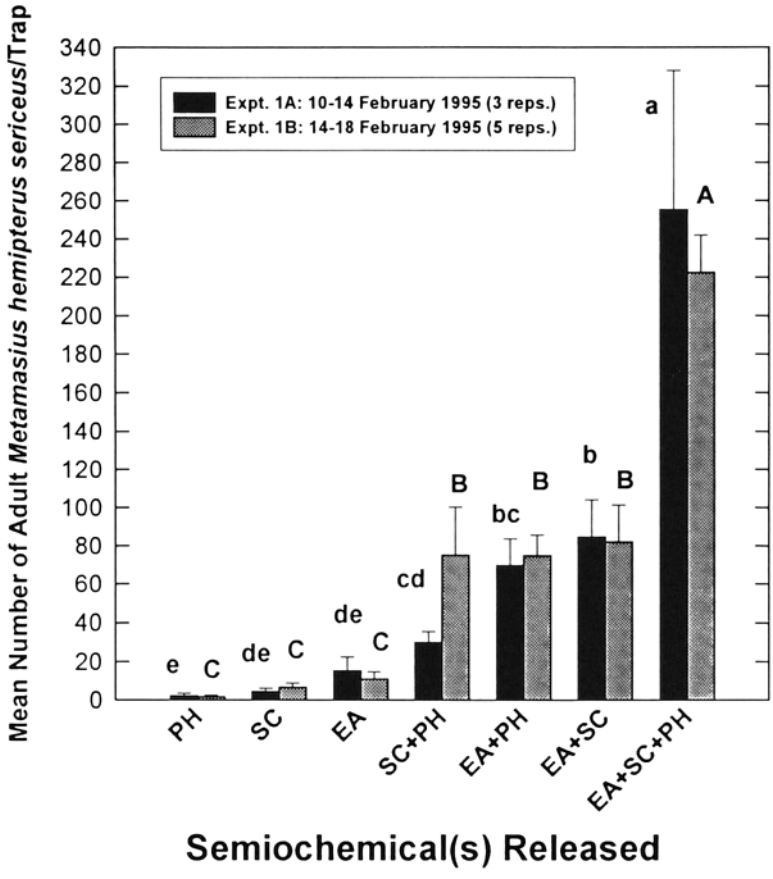

FIG. 3. Comparison of counts of *Metamasius hemipterus sericeus* adults (mean ± SE) from lethal trap design A in a Canary Island date palm planting in Broward County, Florida. Traps were baited with 250 g of sugarcane (SC), ethyl acetate (EA, mean release rate for time replicates 1;2) (683;675 mg/day), or metalure (PH) [(±)-5-methyl-nonan-4-ol and (±)-2-methyl-heptan-4-ol, 8:1 ratio, total release rate of 3 mg/day] in all individual, binary, and ternary combinations. Means followed by the same upper- or lowercase letter are not significantly different (least significant difference, $P \leq 0.05$). Count data were $(x + 0.5)^{0.5}$ transformed prior to analysis; untransformed means are presented.

sugarcane weevils in the subfamily Rhynchophorinae suggests that in addition to ethyl acetate, ethyl propionate, and ethyl butyrate, there are probably other classes of unidentified host kairomones that may be shared by these insects (Giblin-Davis et al., 1994a; Gries et al., 1994; Jaffé et al., 1993).

Experiment 2: Field Attraction to Ethyl Acetate or its Hydrolysis Products. Total counts from traps (design A) baited with ethyl acetate (4DH device; 777 ± 39 mg/day) and metalure were greater than traps baited with ethyl acetate hydrolysis products (ethanol and/or acetic acid) and metalure or metalure alone ($F = 30.88$; $df = 4, 4$; $P = 0.0001$) (Figure 4). There was no difference in the proportion of females per trap (50%) for the different treatments ($F = 0.97$; $df = 4, 4$; $P = 0.4840$).

We estimated that under the conditions of this study with ethyl acetate released from the 4DH device that the hydrolysis products, ethanol and acetic acid, would each be released at about 6–8 mg/day (Oehlschlager et al., unpublished data). This experiment (Figure 4) demonstrates that ethanol and/or acetic acid when released at these rates with metalure were not any more attractive than metalure alone. Thus, only ethyl acetate appears to increase trap efficacy.

Experiment 3: Ethyl Acetate Dose-Response with Sugarcane (250 g) and Metalure. Release of ethyl acetate increased efficacy of the semiochemical-based trap (design A) baited with 250 g of sugarcane and metalure in a dose-dependent manner for both males and females of *M. h. sericeus* ($F = 10.93$ and 24.13; $df = 3, 6$ and $3, 6$; $P = 0.0003$ and 0.0001 for females and males, respectively) (Figure 5). Weevil counts increased sharply with up to 400 mg/day of ethyl acetate, then appeared to level off through 1663 mg/day (Figure 5). There was no difference in the proportion of females per trap for the different treatments (44%) ($F = 1.24$; $df = 3, 6$; $P = 0.3258$).

Thus, ethyl acetate, which may be available in nature at much lower concentrations than 400–1600 mg/day (Samarajeewa et al., 1981), is not repellent to *M. h. sericeus* at up to 1663 mg/day. Samarajeewa et al. (1981) showed that the ethyl acetate concentration of arrack, a palm wine distillate, varied between 14 and 3353 mg/liter. Because arrack is a distillation product of palm wine (toddy), the actual concentrations of ethyl acetate in the naturally fermented palm sap would be about three- to seven-fold diluted (i.e., 2–280 mg/liter). Thus, the actual available ethyl acetate to the environment from 250 g of fermenting sugarcane would be contingent upon the microbes present and probably be in the range of 0–30 mg/day. reported that ethyl acetate, ethyl propionate, or ethyl butyrate released alone at about 20–30 mg/day did not synergize attraction of *M. h. sericeus* to metalure at the same level as fermenting sugarcane in Costa Rica. Our work in Florida demonstrates that ethyl acetate released from the 4DH device, which released between 400 and 900 mg/day, depending upon temperature, significantly added to the attraction of metalure or sugarcane in a binary trap or metalure and sugarcane in a ternary trap (Figure 3). Because ethyl acetate is not repellent at the high rates of 400–1600 mg/day in the field (Figure 5), it may increase the effective range of a trap, improve short-range resolution for trap location, or function somehow as a more effective arrestant for *M. h. sericeus* in or near traps. Although ethyl acetate release rates of 400–1600 mg/

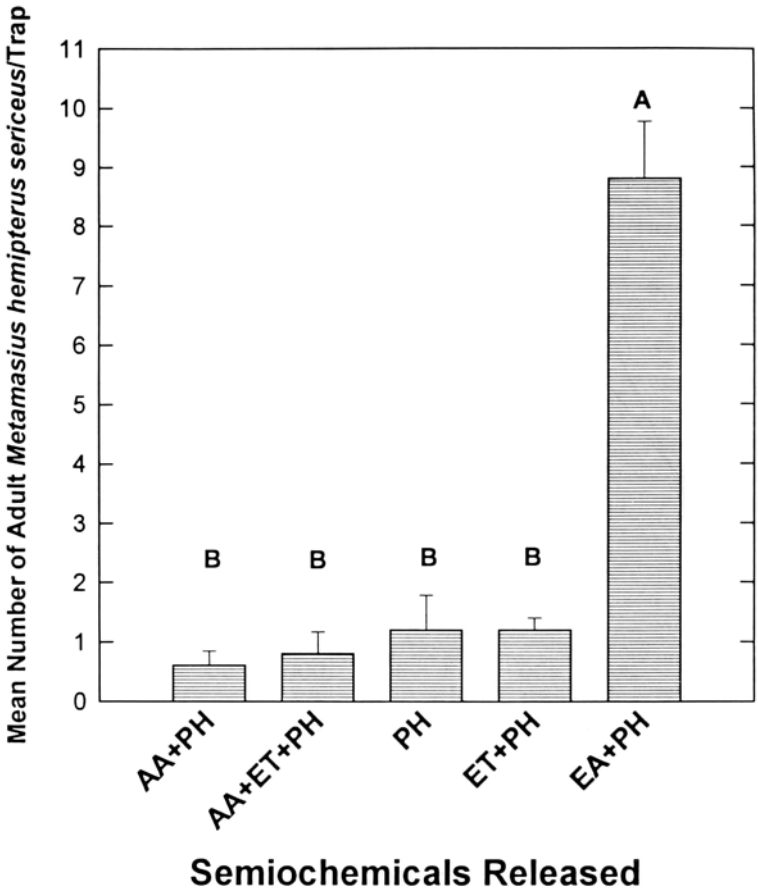

FIG. 4. Comparison of counts of *Metamasius hemipterus sericeus* adults (mean ± SE) from lethal trap design A in a Canary Island date palm planting in Broward County, Florida (March 7–13, 1995, five replicates). Traps were baited with metalure (PH) [(±)-5-methyl-nonan-4-ol and (±)-2-methyl-heptan-4-ol, 8:1 ratio, total release ratio of 3 mg/day] alone, or with ethyl acetate (EA) (777 mg/day), acetic acid (AA) (6–8 mg/day), ethanol (ET) (6–8 mg/day), or AA and ET. Means followed by the same uppercase letter are not significantly different (least significant difference, $P \leq 0.05$). Count data were $(x + 0.5)^{0.5}$ transformed prior to analysis; untransformed means are presented.

day may seem high relative to 250 g of sugarcane, these rates may not be that high relative to the local levels produced in heavily rat- or wind-damaged sugarcane fields or in large damaged palm trees. In addition, Jaffé et al. (1993) suggested that ethyl acetate is perceived by males of *R. palmarum* and stimulates

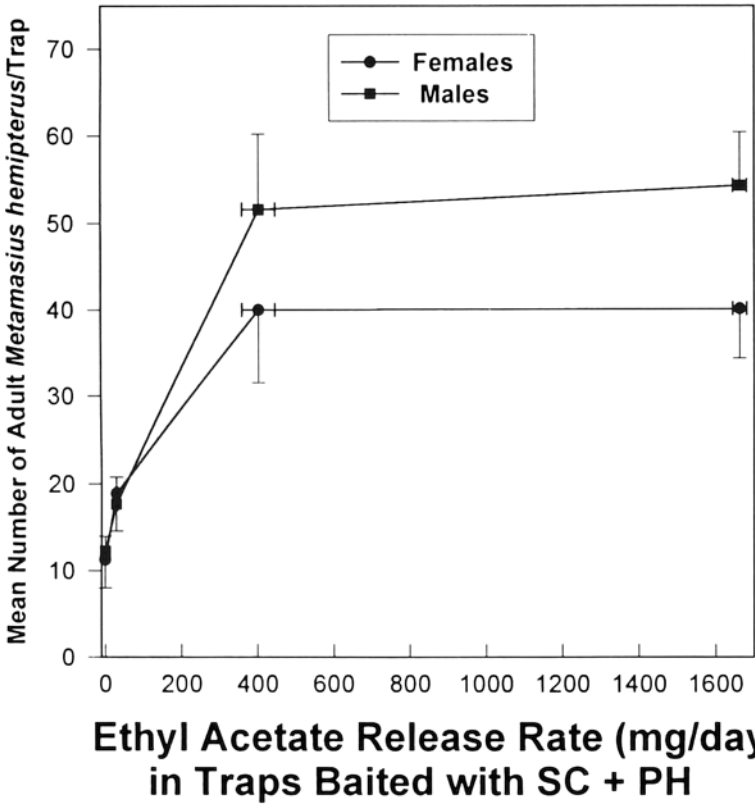

FIG. 5. Plot of counts of *Metamasius hemipterus sericeus* females and males (mean ± SE) from lethal trap design A in a Canary Island date palm planting in Broward County, Florida (February 22–27, 1995) (seven replicates). Traps were baited with 250 g of sugarcane (SC) and metalure (PH) [(±)-5-methyl-nonan-4-ol and (±)-2-methyl-heptan-4-ol, 8:1 ratio, total release rate of 3 mg/day], with different release rates of ethyl acetate (0, 29, 403, 1663 mg/day).

them to produce their aggregation pheromones. If this is true for *M. h. sericeus*, then live weevils in the vicinity of a trap baited with high levels of ethyl acetate might produce more pheromone than weevils in the vicinity of traps with little or no ethyl acetate. Recent work with metalure release rates and *M. h. sericeus* field attraction (Oehlschlager et al., unpublished data) suggests that a 10-fold increase in metalure with sugarcane results in a doubling of trap counts in Costa Rica. However, this modest increase in trap efficacy does not outweigh the increased cost of the metalure. Thus, some of the effect of ethyl acetate may be

due to stimulation of weevils to produce aggregation pheromones prior to entry into the trap.

Experiment 4: Sugarcane Dose–Response with Ethyl Acetate (4DH device; 400–800 mg/day) and Metalure. Regarding 0–250 g sugarcane, male and female counts from traps (design A) baited with ethyl acetate, metalure, and 250 g of sugarcane were greater than traps baited with ethyl acetate, metalure, and 100, 50, or 0 g of sugarcane ($F = 39.83$ and 17.60; $df = 3, 6$ and 3, 6; $P = 0.0001$ and 0.0001 for females and males, respectively) (Figure 6). There was no difference in the release rates of ethyl acetate from the different treatments; mean ethyl acetate release rate was 509 mg/day ($F = 0.63$; $df = 3, 6$; $P = 0.6074$). There also was no difference in the proportion of females per trap (38%) for the different treatments ($F = 0.37$; $df = 3, 6$; $P = 0.7764$). This experiment demonstrates that 250 g of sugarcane provides enough of an increase in trap counts to justify the time and expense of using it over lesser amounts.

Regarding 250–2000 g sugarcane, female counts from traps (design D) baited with ethyl acetate, metalure, and 2 kg of sugarcane were greater than counts from traps baited with ethyl acetate, metalure, and 500 or 250 g of sugarcane, which were equal to each other ($F = 23.80$ and 7.24; $df = 2, 6$ and 2, 6; $P = 0.0001$ and 0.0087 for females and males, respectively) (Figure 7). Male counts from traps baited with ethyl acetate, metalure, and 2 kg of sugarcane were greater than counts from traps baited with ethyl acetate, metalure, and 250 g of sugarcane; male counts from traps with 500 g of sugarcane were intermediate to traps baited with 250 or 2 kg of sugarcane (Figure 7). There was no difference in the release rates of ethyl acetate from the different treatments; mean ethyl acetate release rate was 652 mg/day ($F = 0.89$; $df = 2, 6$; $P = 0.4389$). There also was no difference in the proportion of females per trap for the different treatments (42%) ($F = 1.06$; $df = 2, 6$; $P = 0.3761$). Ethyl acetate was released in this experiment within the range of its highest effective dose (400–1600 mg/day), and larger amounts of sugarcane continued to increase trap counts of *M. h. sericeus*. This supports the hypothesis that other class(es) of kairomones still need to be identified from fermenting host tissue. The added number of weevils captured due to the increase of sugarcane per trap from 250 g to 2 kg probably does not justify the added cost.

Experiment 5: Sugarcane Substitutes. Male counts from traps (design D) baited with ethyl acetate, metalure, and 250 g of sugarcane were equal to counts from traps baited with ethyl acetate, metalure, and a cotton terry towel soaked with molasses and water (with or without yeast) (Figure 8). Male counts from traps baited with ethyl acetate, metalure, and molasses (with no water) were intermediate to counts from traps baited with ethyl acetate, metalure, and molasses and water (with or without yeast) and to counts from traps baited with ethyl acetate and metalure only, but lower than counts from traps baited with ethyl acetate, metalure, and sugarcane ($F = 5.82$; $df = 4, 4$; $P = 0.0043$) (Figure

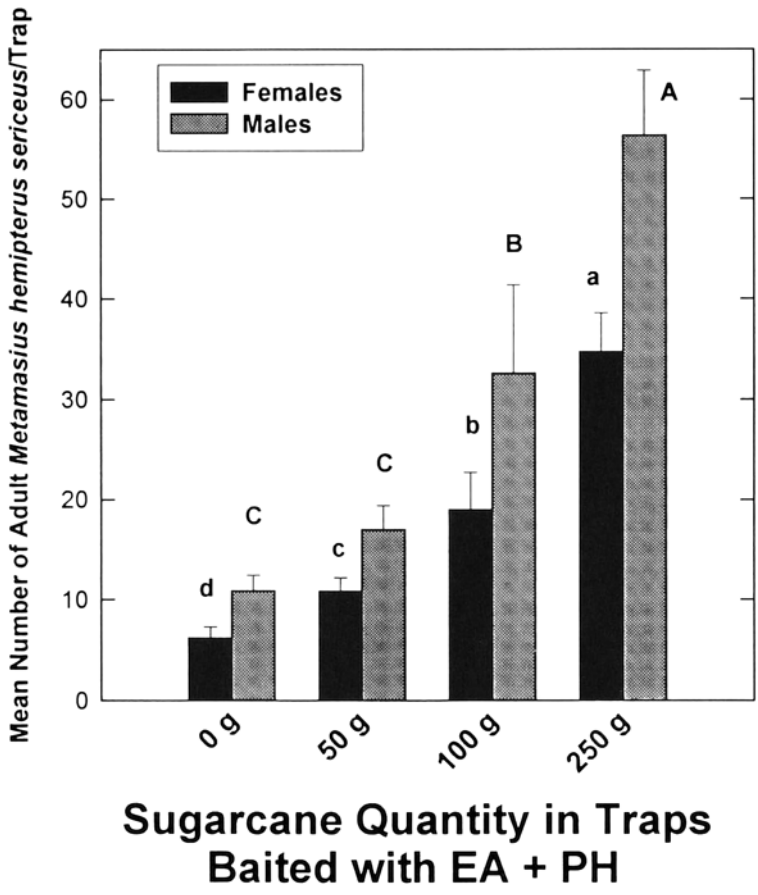

FIG. 6. Comparison of counts of *Metamasius hemipterus sericeus* females and males (mean ± SE) from lethal trap design A in a Canary Island date palm planting in Broward County, Florida (March 13–17, 1995) (seven replicates). Traps were baited with ethyl acetate (EA) (509 mg/day) and metalure (PH) [(±)-5-methyl-nonan-4-ol and (±)-2-methyl-heptan-4-ol, 8:1 ratio, total release rate of 3 mg/day], with different amounts of sugarcane (0, 50, 100, or 250 g). Means followed by the same upper- or lowercase letter are not significantly different (least significant difference, $P \leq 0.05$). Count data were $(x + 0.5)^{0.5}$ transformed prior to analysis; untransformed means are presented.

8). Female counts showed similar trends to the males ($F = 7.10$; $df = 4, 4$; $P = 0.0017$) (Figure 8). The molasses treatment without water smelled more like molasses than both the molasses and water treatments that had a fermented smell. The pH of the fluid from the towels of both molasses and water treatments (with and without yeast) were similar, at about 2.3, after five days in the field. The

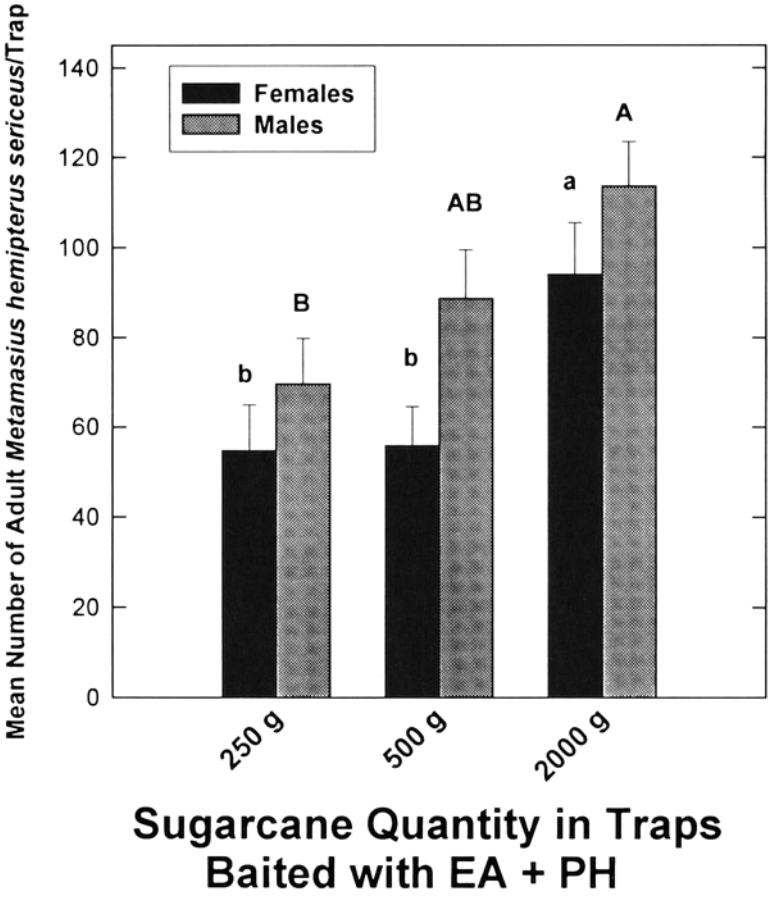

FIG. 7. Comparison of counts of *Metamasius hemipterus sericeus* females and males (mean ± SE) from lethal trap design D (modified with a 4.9-liter stimulus container) in a Canary Island date plam planting in Broward County, Florida (March 22–27, 1995) (seven replicates). Traps were baited with ethyl acetate (EA) (652 mg/day) and metalure (PH) [(±)-5-methyl-nonan-4-ol and (±)-2-methyl-heptan-4-ol, 8:1 ratio, total release rate of 3 mg/day], with different amounts of sugarcane (250, 500, and 2000 g). Means followed by the same upper- or lowercase letter are not significantly different (least significant difference, $P \leq 0.05$). Count data were $(x + 0.5)^{0.5}$ transformed prior to analysis; untransformed means are presented.

treatment with yeast smelled more yeasty than any of the other treatments. There was no difference in the release rates of ethyl acetate from the different treatments; total mean ethyl acetate release rate was 847 mg/day ($F = 0.47$; $df = 4, 4$; $P = 0.7586$). There was no difference in the proportion of females per

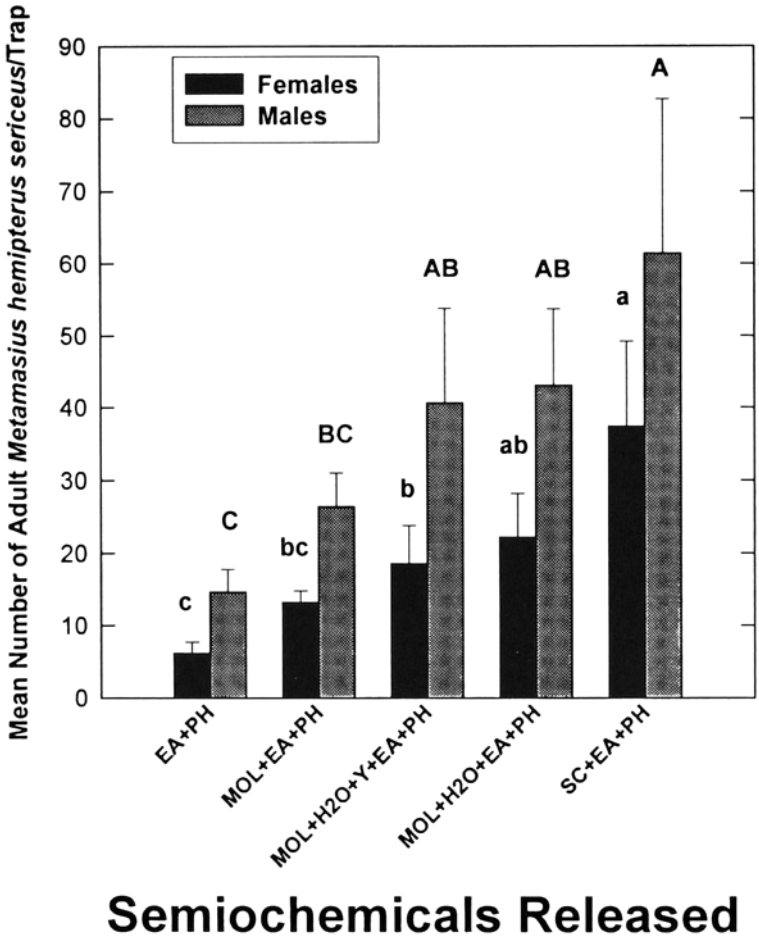

FIG. 8. Comparison of counts of *Metamasius hemipterus sericeus* females and males (mean ± SE) from lethal trap design D in a Canary Island date palm planting in Broward County, Florida (five replicates). Traps were baited with ethyl acetate (EA) (847 mg/day) and metalure (PH) [(±)-5-methyl-nonan-4-ol and (±)-2-methyl-heptan-4-ol, 8:1 ratio, total release rate of 3 mg/day] only, or with 250 g of sugarcane (SC), a cotton towel with 45 g of molasses (MOL), MOL and 158 ml of tap water (H_2O), or MOL + H_2O and 0.15 g of Fleischman's yeast (Y). Means followed by the same upper- or lowercase letter are not significantly different (least significant difference, $P \leq 0.05$). Count data were $(x + 0.5)^{0.5}$ transformed prior to analysis; untransformed means are presented.

trap for the different treatments (34%) ($F = 0.58$; $df = 4, 4$; $P = 0.6815$). The results from our experiment corroborate work by Arango and Rizo (1977), where a yeast, water, and molasses mixture that was applied to sugarcane bagasse in single open-ended bamboo cylinder traps with pesticide was shown to be attractive to *M. hemipterus* in Colombia. The data also show that water is necessary for fermentation of molasses to produce kairomones for *M. h. sericeus*.

Fermenting sugarcane and pineapple work equally as well for attracting *M. h. sericeus* (Giblin-Davis et al., 1994b). However, these materials can be expensive or unavailable in parts of Florida. In addition, storing cut sugarcane for trapping can attract unwanted weevils into an area. This experiment (Figure 8) shows that molasses and water can be used as an inexpensive and easily obtained substitute for sugarcane or pineapple for future work with semiochemical-based trapping of *M. h. sericeus*.

Optimization of Trap Design, Placement, and Color Using Sugarcane (250 g), Ethyl Acetate (4DH Release Device), and Metalure as Bait

Design. Counts from trap designs B–D baited with the ternary semiochemical combination [ethyl acetate (4DH device), sugarcane (250 g), and metalure] were equal and greater than counts from trap design A ($F = 4.58$ and 1.39; $df = 3, 6$ and 3, 6; $P = 0.0159$ and 0.2813 for females and males, respectively) (Figure 1). There was no difference in the release rates of ethyl acetate from the different trap designs: mean release rate of 672 mg/day ($F = 0.65$; $df = 3, 6$; $P = 0.5936$). There was no difference in the proportion of females per trap (55%) for the different trap designs ($F = 0.63$; $df = 3, 6$; $P = 0.6032$).

It is not clear why trap A performed less effectively than traps B–D. Traps B–D have more surface area for landing and crawling than design A. Trap B had the greatest surface area and connection to the soil. Trap C is similar in design to the trap used in Costa Rica for *M. h. sericeus* (Perez et al., 1995) and *R. palmarum* (Oehlschlager et al., 1993), except that it was not hung on palms at chest height, allowing the palm to function as a landing and walking platform. *Rhynchophorus palmarum* required trap contact with the ground or palm trunk for significant trap efficacy (Oehlschlager et al., 1993). The relatively small *M. h. sericeus* is more agile in flight than the larger species of *Rhynchophorus* and therefore may not require a large landing surface or connection to the soil. However, differences in the amount of suitable landing surface area between trap A and traps B–D may account for the lower trap efficacy in trap A.

Height. Weevil counts from trap design D baited with the ternary semiochemical combination [ethyl acetate (4DH release device), sugarcane (250 g), and metalure] at 1 m on a pole and on the ground were equal ($T = -1.56$ and -1.48; $df = 18$ and 18; $P = 0.1353$ and 0.1574 for females and males,

respectively). Traps on the ground caught 76 ± 10 females and 118 ± 17 males compared with 57 ± 7 females and 86 ± 12 males at 1 m. This suggests, as observed for *R. palmarum* (Oehlschlager et al., 1993), that traps with greater connection to possible landing areas may work a little better. However, because *M. h. sericeus* appears to be more agile in flight than *R. palmarum*, it can still enter a trap even when it is suspended in the air on a pole. There was no significant difference in the release rates of ethyl acetate from the different trap designs: mean release rate of 758 mg/day ($T = -0.22$; $df = 18$; $P = 0.8211$). There was no difference in the proportion of females per trap (40%) for the different trap heights ($T = 0.07$; $df = 18$; $P = 0.9455$). We have had problems with the ground-mounted trap (design D) in subsequent work because raccoons learned how to get into them and eat the weevils. This has not yet occurred with the pole mounted trap design D.

Color. Weevil counts from black, white, and gray ground-mounted traps (design D) that were baited with the ternary semiochemical combination [ethyl acetate (4DH device), sugarcane (250 g), and metalure] were equal ($F = 1.09$ and 1.48; $df = 2, 4$ and 2, 4; $P = 0.3813$ and 0.2846 for females and males, respectively). Black, white, and gray traps caught 43 ± 11, 32 ± 11, and 28 ± 5 females and 45 ± 11, 32 ± 11, and 38 ± 10 males, respectively. There was no difference in the release rates of ethyl acetate from the different trap colors: mean release of rate 796 mg/day ($F = 1.47$; $df = 2, 4$; $P = 0.2851$). There was no difference in the proportion of females per trap (48%) for the different trap designs ($F = 0.37$; $df = 2, 4$; $P = 0.7037$).

Weevil counts from black, blue, green, red, and yellow ground-mounted traps (design D) that were baited with the ternary semiochemical combination [ethyl acetate (4DH device), sugarcane (250 g), and metalure] were equal ($F = 1.24$ and 0.12; $df = 4, 4$ and 4, 4; $P = 0.3352$ and 0.9742 for females and males, respectively). Black, blue, green, red, and yellow traps caught 28 ± 4, 18 ± 5, 21 ± 5, 25 ± 6, and 18 ± 4 females and 40 ± 4, 42 ± 4, 45 ± 7, 44 ± 6, and 44 ± 19 males, respectively. There was no difference in the release rates of ethyl acetate from the different trap colors: mean release rate of 825 mg/day ($F = 2.10$; $df = 4, 4$; $P = 0.1282$). There was no difference in the proportion of females per trap (33%) for the different trap colors ($F = 2.44$; $df = 4, 4$; $P = 0.0894$). Similarly, Oehlschlager et al. (1993) reported for *R. palmarum* that there was no difference in semiochemical-based trap efficacy with different trap colors.

CONCLUSIONS

Our study demonstrates that in banana and palm plantings in Florida, the kairomone ethyl acetate was as effective at 500–1000 mg/day as 250 g of fer-

menting sugarcane alone for capturing *M. h. sericeus*. This led to a study of the effects of ethyl acetate, metalure, and/or 250 g of sugarcane alone or in binary or ternary combinations on trap counts of *M. h. sericeus*. Each of these semiochemicals or semiochemical sources were additive or synergistic. It was ethyl acetate and not its hydrolysis products (ethanol and acetic acid) that were attractive to *M. h. sericeus*. Ethyl acetate increased attraction to traps baited with sugarcane and metalure in a dose-responsive manner with the most attraction occurring between 400 and 1600 mg/day. Increases in the amounts of fermenting sugarcane in traps baited with ethyl acetate and metalure resulted in increases in trap efficacy. So far, the most cost-effective semiochemical-based bait is a ternary combination of sugarcane (250 g), ethyl acetate (400–800 mg/day), and metalure (3 mg/day). Molasses (45 g) and water (158 ml) can be used as a substitute for 250 g of sugercane in situations where sugarcane is not available or is too expensive. Physical trap designs were studied, and it appears that traps with greater surface area work better. Trap placement (on ground vs. 1 m high) and color of trap body do not appear to affect trap efficacy.

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LIPID COMPOUNDS IN SECRETIONS OF FISHING BAT, *Noctilio leporinus* (CHIROPTERA: NOCTILIONIDAE)

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Abstract—The distinctive odor of *Noctilio leporinus* arises from oily secretions found beneath the wings in the subaxillary region. Analysis of secretions by gas chromatography–mass spectrometry identified 372 lipid compounds. Differences in number and chemical composition of glycolipids suggest that secretions of males from the same roost are more similar to each other than to other males or females. Differences in number and chemical composition of nonpolar lipids indicate that secretions of males are more similar to each other than to females. Since secretions differ between sexes, information on sexual identity and reproductive condition may be communicated. Individually unique lipid compositions further suggest that bats may be recognizable by their odor within the roost and while flying.

Key Words—Bats, scent marking, lipids.

INTRODUCTION

Bats (Order Chiroptera), like other mammals, produce an assortment of scents that are used in intraspecific communication (Schmidt, 1985). Although distinctive odors are common in many bat species, the function of most of these scents is poorly known. Individual identification from olfactory signals has been suggested by research on groups of little brown bats (*Myotis lucifigus*) (Thomas et al., 1979) and for individual vampire bats (*Desmodus rotundus*) (Schmidt and Manske, 1973). Mother Mexican free-tailed bats (*Tadarida brasiliensis*) use olfaction to help distinguish their young in densely packed nursery creches that

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contain tens of thousands of similarly aged young (McCracken and Gustin, 1991).

A few instances are known of bats using secretions to mark territories. Harem boundaries are delineated by male gray-headed flying foxes (*Pteropus poliocephalus*) (Nelson, 1965) and insular fruit bats (*P. tonganus*) (Grant and Banack, 1995) by rubbing secretions from neck and shoulder glands against tree branches. Both male and female Samoan flying foxes (*P. samoensis*) mark vegetation within foraging areas with secretions from neck glands (Brooke, personal observation).

Bats also use secretions to mark conspecifics. Dominant male velvet free-tailed bats (*Molossus molossus*) mark both females and subordinate males with musky smelling secretions from their throat glands (Schmidt, 1985). Displaying male sac-winged bats, *Saccopteryx bilineata*, shake their wings at females and rival males dispensing secretions from wing pockets (Bradbury and Emmons, 1974).

Increases in secretions corresponding with seasonal mating have been found in male gray-headed flying foxes, *P. poliocephalus* (Ratcliff, 1932; Herreid, 1960; Nelson, 1965), velvet free-tailed bats, *M. molossus* (Schmidt, 1985), and Mexican free-tailed bats, *T. brasiliensis* (Davis et al., 1962), suggesting that scent production is a secondary sexual signal. In addition, sexually dimorphic chest glands are present in the Phyllostomidae (Valdivieso and Tamsitt, 1964) and Molossidae (Werner and Lay, 1963).

The fishing bat, *Noctilio leporinus*, has a particularly strong and distinctive odor. Goodwin (1928) identified flying *N. leporinus* on nights too dark to make visual observations on the basis of their distinctive musky odor. This smell has been attributed to their fish-eating diet (Gudger, 1945; Goodwin and Greenhall, 1961) or, alternatively, to the rank odor from the inguinal glands (Ruschi, 1951; Studier and Lavoie, 1984). Two different smells present are produced in different places: the skin and hair of the subaxial area beneath the wings of both males and females is covered by varying amounts of an oily yellow fluid that had a strong, musky-sweet odor, and second in males there is a rank or bitter scent that comes from the inguinal pockets found on either side of the testes.

Subaxial secretions have been reported only in one other bat species, the smaller *N. albiventris* (Dunn, 1934). In both *N. albiventris* and *N. leporinus* the musky-sweet odor is stronger and more noticeable in males than in females (Goodwin, 1928; Dunn, 1934; Gudger, 1945). The odor is persistent: people who have handled bats of either species reported that the distinctive odor remains on their hands for several days, despite removal efforts (Dunn, 1934; Goodwin, 1928). No obvious gland can be found in the subaxial region of either species (Dunn, 1934; Brooke, personal observation).

Noctilio leoprinus has a polygynous social organization where discrete groups of 3–10 females cluster together with a single harem male in the roost

(Brooke, 1994). Many groups may utilize the same hollow tree or rock crevice, but each group exclusively uses a specific area within the roost. Female groups may remain together in the same location for several years regardless of turnover in harem males, who may retain their tenure for two or more reproductive seasons. Bachelor males roost alone or in small groups separate from females.

Female bats marked themselves with the subaxial secretions of other females in the same group (Brooke, 1994). "Head rubs" occurred when a female pressed her head beneath the wing of another and rotated her head from side to side, thereby transferring the scent of the other bat. Females also rubbed their foreheads or chins against the foreheads of other females in their group. Females did not rub against either the subaxial area of resident males or the males' head. Female bats routinely "visited" different groups within the roost (Brooke, 1994). When a visitor first entered the group of roosting bats, her head and wings were thoroughly smelled by the groups. Conversely, group members did not elicit any interest when entering their group.

In this paper we investigate whether the scent of *N. leporinus* differs between adult males and females. Bats were captured to measure the amount of secretions and collect a sample for analysis. Secretions were collected and analyzed to determine differences in the chemical compounds between individuals, within social groups, and among different roosts. We compare lipids of adult males and females and of individuals from different roosts. Bats of known sex and social status were followed while foraging and when entering and exiting a night roost to detect odor in flight.

METHODS AND MATERIALS

This study was conducted on the island of Culebra, 40 km east of Puerto Rico. Culebra is approximately 11 km × 3 km at its longest and widest points, respectively (for a complete description of the study site see Brooke, 1994).

Bats were captured in mist nets while exiting or returning to their day roost. Relative age, adult or juvenile (less than 1 year old) was determined by tooth wear and fused epiphysial joints (Brooke, 1994). The area covered by subaxial secretions was measured in square millimeters with a ruler.

Samples of subaxial secretions from 12 individuals (five males and seven females) from three different roosts were collected on sterile swabs, sealed in glass containers, and frozen. Secretions were extracted by a modified Bligh-Dyer solvent system (While et al., 1979) by D. Decker. Samples were analyzed for lipid composition following methods described for extraction, lipid isolation, gas chromatography, and mass spectrometry in Decker et al. (1992). The nonpolar lipid sample from one female was lost.

To determine how the lipid components of secretions varied among indi-

viduals, sexes, and different roosts, the presence and quantities (in picomoles) of each lipid compound were compared using an unweighted paired-group cluster analysis using arithmetic averages (UPGMA) using the NTSYS computer program (Rohlf, 1987).

To establish differences between males and females in flight, we followed two harem males and 23 females by radio telemetry (Brooke, 1994). On 10 occasions when bats flew within 5 m of us, we noted whether the distinctive smell was evident. Behavioral observations were made inside a building used as a night roost by up to 30 bats during May 1989. Whenever bats entered the building and flew from the roost we noted whether we could detect the musky odor. Sex of bats was established when each roosted.

RESULTS

The area covered by subaxial secretions was determined in 199 bats. The area covered by secretions in adults varied among individuals and between the sexes. There were no visible secretions on 43 juvenile bats examined. Of the 80 adult females examined, 59 individuals (73%) had no visible subaxillary secretions, 11 (14%) had only trace amounts visible, and 10 (12%) had 15–100 mm^2 covered by secretions. None of the 59 females without secretions had the sweet odor typical of males, while the 21 females with visible secretions carried the odor. Of the 76 adult males examined, 22 individuals (29%) had no visible secretions, while 54 (71%) had up to 200 mm^2 covered by secretions. Only males with secretions carried the distinct odor.

A total of 372 lipid compounds was identified in the secretions of 13 individuals: 52 glycolipids (Table 1), 196 nonpolar lipids (Table 2), and 124 phospholipids. Males and females shared 45 glycolipid compounds, 95 nonpolar compounds, and 13 phospholipid compounds. Males had an additional five glycolipid compounds, 55 nonpolar lipid compounds, and 13 phospholipid compounds not shared with females. Females had two glycolipid compounds, 46 nonpolar lipid compounds and 40 phospholipid compounds not shared with males. Three glycolipid compounds (12, 26, and 40) were found in large quantities in both sexes (Table 1).

The UPGMA showed differences by sex and roost affiliation in glycolipid (Figure 1) and nonpolar lipid fractions (Figure 2). Since there was no apparent separation of individuals by either sex or roost affilitation by phospholipids, we have not reported further on these samples. There was no clear separation by sex within the glycolipid analysis. Harem males from the same roost (two individuals from roost 1 and two individuals from roost 3) were more similar to each other than to any other individual (Figure 1). Glycolipid results from a third male, however, were closer to females than to any of the other males.

TABLE 1. RELATIVE QUANTITIES IN PICOMOLES OF GLYCOLIPID COMPOUNDS IN 5 MALE AND 8 FEMALE *Noctilio leporinus*

		Male					Female							
#	Compound	775	764	126	328	712	218	229	781	760	761	327	777	778
1	12:0	0	0	0	5,121	0	0	0	453	1,478	0	631	0	0
2	Phenol	126	0	3,886	3,986	870	0	1,331	853	135	0	0	0	398
3	Thiocyanic acid	0	0	0	0	8,517	0	0	550	841	0	0	0	0
4	Unknown	0	1,187	0	0	0	0	0	1,030	619	0	0	0	0
5	14:0	30	0	682	686	487	0	758	918	0	0	0	0	0
6	Unknown	391	2,849	3,200	4,096	3,329	0	0	0	0	0	0	0	0
7	15:1	155	2,891	1,237	1,774	699	941	706	723	3,250	19,092	0	768	740
8	Hydrocarbon	0	0	1,863	1,473	1,157	0	1,097	1,546	929	0	954	0	0
9	18:2	54	0	0	0	0	0	0	0	5,295	0	0	0	0
10	16:1w9c	171	0	0	0	0	0	0	3,991	3,848	0	0	0	0
11	16:1w7c	521	35,537	17,280	27,666	11,358	23,527	14,445	36,900	42,418	143,818	13,090	9,123	16,225
12	16:0	40,226	423,287	318,740	0	343,016	389,156	328,353	396,261	439,635	5,055,036	424,457	349,191	437,672
13	Unknown	329	0	5,941	10,583	12,142	8,523	10,641	3,222	4,023	0	10,191	10,675	0
14	17:2	186	3,777	1,278	1,514	0	890	0	1,221	627	18,604	0	0	0
15	a17:0	1,509	25,665	6,529	15,525	1,725	9,665	6,970	7,017	10,628	133,804	4,954	5,024	8,329
16	17:1	1,295	0	1,838	9,305	5,230	5,112	2,515	6,783	12,488	0	4,873	0	0
17	17:1w8	350	0	0	6,132	0	0	0	2,021	0	0	0	0	6,176
18	Hydrocarbon	0	3,146	0	0	0	0	0	0	0	0	0	0	0
19	17:0	2,266	15,680	19,507	26,264	9,924	23,723	0	22,681	21,245	368,545	26,427	28,227	2,323
20	Unknown	292	1,671	2,117	4,151	1,872	0	22,869	1,028	2,909	0	4,463	0	0
21	18:2w6	1,364	0	0	227,480	0	0	11,489	18,473	18,473	0	9,132	0	0
22	18:3w3	2,887	69,814	18,678	0	15,587	16,863	0	6,704	8,981	229,387	0	9,039	15,670
23	18:1w9c	2,942	19,630	28,783	49,000	27,821	17,795	13,002	14,657	26,474	198,795	32,217	37,788	35,928
24	18:1w7t	721	0	7,035	14,145	9,647	4,799	11,323	3,262	5,981	19,226	12,623	10,695	0
25	18:1w5c	0	0	0	0	0	7,435	0	62,861	69,283	0	74,110	0	8,026
26	18:0	10,191	0	201,387	228,795	281,829	204,820	316,500	164,887	116,265	3,330,835	202,077	322,919	214,424

TABLE 1. Continued

		Male					Female							
#	Compound	775	764	126	328	712	218	229	781	760	761	327	777	778
27	18:2	1,002	0	3,219	4,039	0	831	2,947	4,694	5,295	17,305	0	1,606	902
28	Unknown	9,536	1,211	2,625	542	0	0	0	330	0	0	0	7,263	0
29	18:2	0	0	0	0	0	0	0	0	120	0	0	0	0
30	18:2	479	8,074	2,876	0	2,227	2,145	3,804	1,014	856	26,376	1,020	0	233
31	19:1	1,398	44,885	9,310	8,772	8,368	14,758	4,493	16,113	6,598	183,676	7,492	0	6,626
32	20:4w6	6,567	67,686	29,900	40,922	24,297	36,407	0	39,942	35,155	320,345	0	14,517	55,816
33	20:2w3	0	79,791	0	0	0	0	0	16,422	23,669	0	23,343	0	0
34	20:5w3	0	0	1,218	0	0	0	0	0	0	0	0	0	0
35	20:2w3	338	7,329	4,115	6,020	7,557	0	10,797	899	2,309	91,100	7,800	7,623	508
36	20:2w6	690	2,910	0	0	0	0	0	0	0	0	0	0	0
37	20:3w3	4,679	80,318	31,945	43,510	21,382	29,083	20,286	13,103	12,687	298,663	14,049	16,452	23,965
38	20:1w9c	1,084	14,945	12,116	15,403	11,929	9,007	6,908	8,416	4,438	134,427	5,222	8,380	5,804
39	20:1w9t	297	3,078	6,499	8,145	3,844	1,844	1,817	0	1,601	29,649	1,113	1,198	1,679
40	20:0	4,178	14,405	108,469	116,827	89,825	64,771	142,941	89,201	45,436	1,370,933	85,560	110,935	81,531
41	Phenol	0	0	4,368	4,681	3,015	0	1,408	1,113	0	0	0	0	0
42	22:6w3	1,315	0	20,692	0	11,293	9,403	23,959	0	0	0	3,317	20,692	17,060
43	22:4w6	60	0	332	0	0	0	0	663	14,452	253,327	398	0	0
44	22:5w3	342	0	1,963	2,376	1,241	714	565	0	987	0	1,049	621	0
45	22:0	2,926	11,758	0	50,582	31,116	0	0	196	52,869	0	0	0	0
46	Unknown	0	0	0	472	0	24,941	0	30,359	0	0	0	0	36,733
47	a29	0	0	1,512	0	674	0	0	0	29,626	0	0	0	0
48	Benzofuran	307	7,588	4,867	4,343	1,185	0	681	0	0	0	568	0	0
49	20:4w6	6,620	0	0	52,775	0	0	0	0	267	0	0	0	0
50	a33	0	42,552	38,483	0	33,279	0	0	0	0	0	0	0	0
51	18:1w5c	0	0	76,332	0	0	46,496	33,443	18,759	0	0	28,012	27,255	28,636
52	20:1w9c	0	0	0	645	0	0	0	0	25,060	0	0	0	0

TABLE 2. RELATIVE QUANTITIES, IN PICOMOLES, OF NONPOLAR LIPID COMPOUNDS IN 5 MALE AND 7 FEMALE *Noctilio leporinus*

	Male					Female					
Compound	775	764	126	328	712	229	781	760	761	327	777	778
1 Unknown	0	0	0	0	0	0	7,160	887	0	1,953	0	0
2 Tetradecanal	0	0	0	0	0	0	157	0	0	0	0	0
3 Unknown	133	0	139	0	250	0	0	0	0	0	0	0
4 Unknown	0	0	352	249	231	0	0	0	0	0	0	0
5 Unknown	0	0	0	100	0	0	0	0	0	0	0	0
6 Unknown	0	298	0	0	0	0	0	0	0	0	0	0
7 Unknown	0	0	0	0	0	0	0	3,170	0	0	0	0
8 Tridecanal	0	8,754	0	0	0	0	3,275	4,355	0	0	0	0
9 Unknown	0	848	0	0	0	0	131	124	0	0	0	0
10 Unknown	0	0	0	0	0	0	0	0	0	2,065	0	0
11 14:0	0	0	0	0	0	0	0	0	2,062	1,442	0	1,145
12 Naphthalene cpd.	0	0	0	0	0	5,203	0	0	9,773	0	12,926	10,882
13 Unknown	7,562	9,892	0	5,946	0	0	17,678	21,566	0	11,448	0	0
14 Unknown	0	0	0	2,160	0	0	0	0	0	0	0	0
15 15:1w	0	0	0	0	0	0	0	154	0	0	0	0
16 Unknown	1,042	2,820	0	906	0	0	488	1,143	0	0	0	0
17 Unknown	0	1,206	0	0	0	0	232	572	0	0	0	0
18 Naphthelene cpd.	0	0	7,457	0	6,332	0	0	0	0	0	0	0
19 Tetradecanal	700	0	204	288	247	0	0	0	0	0	0	0
20 Unknown	0	1,085	0	0	0	0	858	975	0	0	0	0
21 15:0	298	0	506	444	675	0	0	0	0	1,731	2,127	0
22 Unknown	0	306	169	0	0	0	0	0	0	0	0	0
23 Unknown	13,563	27,835	0	10,547	12,140	8,692	19,987	24,417	12,530	15,211	16,685	15,110
24 Unknown	0	540	0	0	0	0	0	0	0	0	0	0
25 Unknown	0	0	0	0	0	0	0	311	0	0	0	0
26 Unknown	165	2,782	0	1,984	3,028	0	5,880	6,081	0	9,470	0	0

TABLE 2. Continued

	Compound	Male					Female						
		775	764	126	328	712	229	781	760	761	327	777	778
27	Unknown	783	0	0	0	0	0	240	541	0	0	0	0
28	116:0	0	0	1,405	0	0	3,790	0	0	2,167	0	8,068	4,169
29	16:1w	0	171	0	0	0	3,901	0	0	0	0	0	0
30	16:1w9	10,865	25,802	0	9,052	8,218	6,883	7,538	11,043	8,452	76,027	9,664	8,459
31	16:1w7	1,690	1,558	0	1,067	0	0	1,890	1,792	0	0	0	0
32	Unknown	0	0	0	0	0	0	0	2,246	5,614	0	7,329	0
33	Unknown	13,583	26,651	0	12,819	13,310	6,802	10,299	14,727	8,921	12,996	14,690	9,626
34	Unknown	0	0	14,472	0	0	0	0	1,635	0	0	0	0
35	Pentadecanal	0	0	2,374	0	0	0	0	1,017	0	0	4,363	3,130
36	Unknown	0	0	0	0	0	0	0	0	235,526	288,683	282,023	337,578
37	Unknown	186,669	5,098	150,900	0	0	0	0	0	0	4,856	0	0
38	Alcohol br16:1	0	0	0	0	0	0	0	0	0	1,176	0	0
39	Unknown	0	297,156	0	154,833	162,650	72,645	349,664	354,935	0	0	0	0
40	15:1w	529	992	0	516	579	0	895	853	0	0	0	0
41	Unknown	10,206	0	6,961	6,934	6,552	7,838	8,622	11,190	10,059	1,405	10,020	9,509
42	17:1w8	0	1,917	0	0	0	0	3,489	1,691	0	0	0	0
43	17:1w9	0	0	0	0	0	0	0	0	0	0	0	0
44	Unknown	2,133	3,974	0	2,012	1,253	0	0	0	0	5,855	0	0
45	17:0	0	0	0	0	0	0	0	0	0	992	0	0
46	17:1w6	8,118	11,415	5,851	5,168	5,058	4,369	2,581	5,060	7,058	0	7,403	3,637
47	Unknown	0	0	1,922	0	0	0	0	0	0	0	0	0
48	Unknown	0	0	0	0	0	0	0	203	0	0	0	0
49	Unknown	0	0	0	0	0	0	920	826	0	0	0	0
50	17:0	0	0	0	0	0	0	12,314	12,044	0	0	0	0
51	Unknown	0	0	0	0	0	0	270	0	0	0	0	0
52	Hexadecanal	213	929	0	311	0	0	0	0	0	0	823	0

53	Unknown	934	0	0	0	0	0	0	0	0	0	0	0
54	Unknown	307	0	199	0	0	0	0	0	0	0	0	0
55	Unknown	8,778	8,846	6,655	6,050	5,944	8,505	0	0	11,787	12,597	14,497	12,559
56	Unknown	2,322	5,730	765	598	0	0	4,665	5,529	0	2,298	0	0
57	1,13-Hexadecadiene	0	0	0	0	0	0	0	246	0	0	0	0
58	18:3w6	0	0	0	0	0	0	0	176	0	0	0	0
59	Unknown	0	0	1,719	1,029	1,944	561	0	0	0	0	0	0
60	Unknown	0	682	0	0	0	0	0	0	0	0	0	0
61	Unknown	0	2,235	0	0	0	0	0	0	0	0	0	0
62	Unknown	87	412	0	0	300	0	0	0	0	0	0	0
63	18:3w3	15,289	8,489	6,083	4,502	7,521	6,622	10,052	8,083	7,755	2,024	3,054	2,283
64	Unknown	43,853	47,204	42,592	42,303	0	0	0	0	0	0	0	45,826
65	18:3w3	0	0	0	0	38,468	48,623	26,845	26,581	44,935	26,895	48,427	0
66	18:1w9c	57,852	85,518	60,697	48,093	46,133	76,153	83,454	58,749	75,665	58,901	87,563	70,633
67	18:1w7c	14,494	11,650	0	25,623	24,098	4,989	4,359	13,331	10,252	19,108	28,091	32,105
68	18:1w7t	527	0	18,969	3,593	0	0	0	0	0	0	0	0
69	Unknown	0	0	1,833	0	33,263	0	0	1,100	0	0	0	0
70	Unknown	0	0	1,076	0	0	2,223	0	1,050	0	0	0	0
71	18:1w5c	182	0	0	841	702	0	1,406	306	0	0	4,301	0
72	18:0	13,976	15,255	19,854	15,152	16,404	22,496	18,426	16,854	36,438	25,941	55,986	28,856
73	br:182w	0	0	216	0	494	0	0	0	0	0	0	0
74	br:182w	0	0	0	0	0	0	1,251	3,318	0	498	0	0
75	Unknown	7,690	0	0	0	0	0	1,151	1,493	0	0	0	0
76	Unknown	446,800	0	0	2,727	0	0	0	0	0	0	0	0
77	Unknown	8,996	0	8,932	0	1,494	10,576	5,184	4,098	8,023	4,822	9,325	8,634
78	19:1q12	0	9,541	0	7,338	0	0	0	0	0	0	0	0
79	19:1w8	0	1,091	864	0	0	0	520	0	0	0	0	0
80	19:1w6	15,030	17,825	15,121	15,631	14,369	14,143	13,376	17,993	20,091	23,242	26,735	12,366
81	Unknown	0	0	0	67,559	66,057	0	0	0	0	0	0	0
82	Unknown	0	0	0	0	0	3,450	0	0	2,146	0	3,209	159
83	Unknown	4,222	8,687	0	2,413	6,640	0	0	0	0	0	3,068	0
84	20:5w3	487	1,984	0	528	470	0	0	0	0	0	0	0
85	Unknown	685	0	399	0	251	0	0	0	0	0	0	0

TABLE 2. Continued

	Compound	Male					Female						
		775	764	126	328	712	229	781	760	761	327	777	778
86	Unknown	0	0	0	0	0	0	4,828	5,237	0	7,419	0	0
87	Cyclopropyl 19	0	0	0	0	0	0	0	0	0	761	0	0
88	Unknown	0	0	0	0	0	0	46,557	63,606	38,571	32,905	0	0
89	Unknown	0	0	0	0	0	0	6,796	5,799	0	8,457	0	0
90	Unknown	44,427	13,858	49,500	47,908	39,977	0	41,747	50,877	0	17,959	0	0
91	Polyunsat. 20:3	1,443	1,539	1,104	989	0	0	0	0	0	0	0	0
92	Unknown	0	0	1,954	0	0	0	0	0	0	0	0	0
93	Unknown	0	0	0	0	1,417	0	581	1,892	0	0	0	0
94	20:4w6	0	0	0	0	0	3,323	0	0	2,420	0	0	2,744
95	Unknown	0	0	0	0	0	9,517	0	0	0	0	0	0
96	Unknown	2,673	1,724	0	1,003	0	772	0	0	1,045	0	0	0
97	Unknown	0	2,124	0	0	431	0	0	0	0	0	0	0
98	20:2w?	0	0	0	0	0	0	0	0	0	0	19,563	4,868
99	Unknown	0	134,709	0	85,832	76,193	85,896	39,445	28,227	62,977	46,971	80,771	72,333
100	20:1w9	0	0	0	0	0	0	3,369	1,643	0	0	0	0
101	21-ane ($C_{21}H_{44}$) MW = 296	257	487	0	200	212	0	165	0	0	1,110	0	0
102	20:0	16,858	19,497	21,800	15,617	16,076	9,667	9,104	8,755	11,903	7,361	17,683	9,565
103	Unknown	0	0	205	0	374	0	397	532	0	0	0	0
104	Unknown	3,070	4,013	0	6,520	5,335	0	0	826	0	0	1,863	0
105	Unknown	0	624	0	0	0	0	0	180	0	0	0	0
106	20:3w3	83,625	1,114	103,943	0	0	6,613	0	0	0	0	0	0
107	20:1w9	7,695	0	13,337	9,513	8,791	0	0	0	0	0	0	0
108	20:1w7	0	0	5,909	0	0	0	0	0	0	0	0	1,201
109	Unknown	177	0	0	161	0	0	0	0	0	0	0	0
110	Unknown	0	0	0	0	0	0	0	0	0	0	0	661
111	Unknown	6,029	0	6,782	5,253	4,878	4,654	2,648	1,964	3,838	3,139	4,742	4,268

112	Unknown	0	0	10,090	0	0	0	0	0	0	0	0	0
113	21:1w	0	4,710	458	0	0	0	0	0	0	0	0	0
114	Unknown	0	0	342	0	0	0	459	227	1,255	0	0	0
115	22:4w6	0	0	5,649	0	0	0	0	0	0	0	0	0
116	Unknown	0	0	113	0	0	0	0	0	0	0	0	0
117	22:4,5 or 6	9,789	10,546	0	8,335	7,365	7,488	3,550	2,809	8,806	5,705	9,674	5,178
118	22:6w3	2,780	4,649	0	2,585	2,809	0	2,534	1,687	2,259	1,615	3,264	0
119	Unknown	535	392	0	235	0	0	0	0	0	0	0	0
120	Unknown	779	1,815	0	720	0	0	0	0	0	0	0	0
121	Unknown	876	0	0	1,033	0	0	0	0	0	0	0	0
122	Unknown	23,244	29,446	0	22,354	23,885	23,159	0	0	16,914	0	23,235	18,485
123	Unknown	5,850	9,826	31,024	7,527	7,559	3,735	3,100	2,121	0	0	0	0
124	Unknown	0	0	0	0	0	0	718	286	0	0	0	0
125	22:2w6	0	0	0	0	0	0	13,727	10,749	0	19,758	0	0
126	22:1w7	0	0	2,633	3,274	3,097	0	0	385	0	0	0	0
127	22:0	0	0	36,778	0	0	0	0	3,063	0	0	0	0
128	22:1w9	0	0	10,316	0	0	0	0	0	0	2,809	0	0
129	Unknown	0	0	305	0	0	0	0	0	0	0	0	0
130	Unknown	1,143	0	0	601	0	0	0	0	0	0	0	0
131	Unknown	391	0	0	0	0	0	0	0	0	0	0	0
132	Unknown	0	0	2,221	0	0	0	0	0	0	0	0	0
133	Unknown	0	0	4,885	0	0	0	0	0	0	0	0	0
134	Unknown	0	0	36,781	0	0	0	0	0	0	0	0	0
135	C_{23}	0	0	0	0	0	0	0	0	0	726	0	0
136	22:0	6,841	8,890	13,011	8,739	9,404	5,188	3,938	0	6,245	4,514	8,990	5,255
137	Unknown	2,335	2,543	0	2,082	2,020	1,952	1,440	1,113	1,715	0	2,023	0
138	Unknown	2,227	1,872	0	1,728	1,735	1,603	1,150	1,039	1,940	2,039	2,077	0
139	Methyl phenyl imidazolidinone	0	0	0	0	0	0	232	346	0	0	0	0
140	23:0	6,309	3,975	0	3,768	20,246	2,453	2,357	1,857	6,159	0	3,575	2,492
141	Unknown	0	0	0	372	281	0	0	0	0	0	0	0
142	Unknown	300	0	0	429	0	0	0	0	0	0	0	0
143	C_{24}	0	0	0	0	0	0	0	0	0	558	0	0

TABLE 2. Continued

	Compound	Male					Female						
		775	764	126	328	712	229	781	760	761	327	777	778
144	15:0	0	0	0	0	0	0	0	0	0	2,174	0	0
145	9-24-sene	11,695	7,135	0	9,963	11,792	13,590	8,482	7,438	13,606	16,128	16,242	13,369
146	24:1w9	21,784	16,147	0	20,134	23,756	17,205	12,376	6,048	8,838	9,609	10,461	8,285
147	24:1 Hydrocarbon	3,767	2,297	8,358	8,981	10,487	1,518	949	5,752	0	4,649	4,735	3,557
148	Unknown	0	0	0	1,363	1,568	5,680	0	11,763	0	0	0	0
149	24:0	25,595	15,641	0	0	0	16,687	15,135	11,925	18,208	16,481	21,478	0
150	Unknown	0	0	0	0	0	0	171	0	0	0	0	0
151	Unknown	711	642	0	1,071	442	626	484	361	2,491	710	0	0
152	Unknown	2,024	0	0	1,876	1,400	1,623	1,050	1,193	0	2,569	2,886	1,223
153	Hydrocarbon	0	0	0	217	0	0	0	0	0	0	0	0
154	Dimethyldiene	1,541	443	4,360	1,440	2,004	2,939	4,158	2,933	10,164	1,315	10,476	6,017
155	Unknown	0	2,726	0	6,080	8,443	5,847	5,272	4,711	7,045	6,539	7,561	7,259
156	Unknown	0	726	0	164	0	0	0	331	0	1,725	0	0
157	Unknown	256	0	1,950	233	1,471	543	1,769	1,936	0	1,382	0	0
158	Unknown	0	0	0	0	1,954	0	0	0	0	0	0	0
159	Unknown	0	0	721	0	476	0	0	0	0	0	0	0
160	Unknown	2,384	0	0	2,403	0	658	288	0	0	0	0	0
161	Unknown	184	0	0	505	0	0	0	0	0	0	0	0
162	Unknown	3,198	0	0	1,952	2,440	3,655	1,993	1,771	3,662	4,124	3,966	3,189
163	Unknown	19,580	5,823	0	0	19,115	19,726	12,254	5,034	10,441	9,237	41,069	8,581
164	Unknown	3,903	939	0	5,336	6,253	0	394	952	0	3,742	4,200	3,546
165	Unknown	0	0	0	0	0	492	0	0	0	0	0	0
166	Unknown	6,988	0	0	7,285	9,179	6,451	5,733	4,598	7,592	7,262	7,763	8,009
167	Unknown	237	0	0	0	0	0	566	524	0	0	0	0
168	Unknown	140	0	0	0	436	0	0	0	0	0	0	0
169	Unknown	116,935	0	136,578	12,867	115,198	220,585	78,348	63,175	0	0	0	0

170	Unknown	2,470	0	434	2,884	2,694	2,233	948	500	0	0	0	0
171	Sterol	0	0	773	0	0	0	0	0	0	0	0	0
172	Unknown	0	0	0	0	0	0	0	0	3,617	862	0	0
173	Unknown	944	0	2,365	710	1,576	0	0	0	4,233	0	2,431	0
174	Unknown	0	0	0	0	0	0	0	0	158,328	70,938	0	149,705
175	Unknown	0	0	0	0	0	0	1,115	875	0	2,273	0	0
176	Unknown	0	0	0	0	71,570	63,899	20,481	13,148	41,488	18,811	41,248	31,145
177	Unknown	66,406	0	101,819	90,363	0	0	0	0	0	0	0	0
178	Unknown	0	0	0	0	0	0	0	0	0	0	2,120	2,685
179	Unknown	2,905	0	3,380	0	269	3,068	1,053	840	2,442	11,788	0	0
180	Unknown	0	0	1,024	0	470	0	0	0	0	0	0	0
181	Unknown	0	0	0	0	0	0	0	1,212	0	0	0	0
182	Unknown	0	0	0	0	0	0	617	410	0	0	0	0
183	Unknown	1,785	0	1,772	1,363	1,177	1,403	675	330	0	0	0	0
184	Unknown	0	0	174	135	183	0	0	0	0	0	0	0
185	Unknown	0	0	0	0	0	0	0	0	0	3,955	0	0
186	Unknown	0	11,713	0	0	668	0	0	0	0	0	0	0
187	Unknown	0	0	0	0	0	0	467	0	0	1,097	0	0
188	Unknown	1,236	3,815	0	0	2,366	1,558	1,320	1,076	0	1,789	2,073	0
189	Unknown	9,436	0	1,747	421	0	12,411	952	5,395	0	6,271	6,334	0
190	Unknown	0	0	941	1,3798	5,673	0	0	693	0	1,399	0	0
191	Unknown	0	0	864	0	0	0	0	0	0	0	0	0
192	Unknown	0	0	0	0	0	0	164	0	0	0	0	0
193	Unknown	415	0	0	211	0	0	136	371	0	0	0	0
194	Unknown	9,426	0	0	9,830	0	19,628	0	0	0	0	6,341	9,692
195	Unknown	0	0	11,591	0	11,016	0	14,789	9,827	0	8,378	0	0
196	Unknown	0	0	300	207	0	0	0	0	0	0	0	0

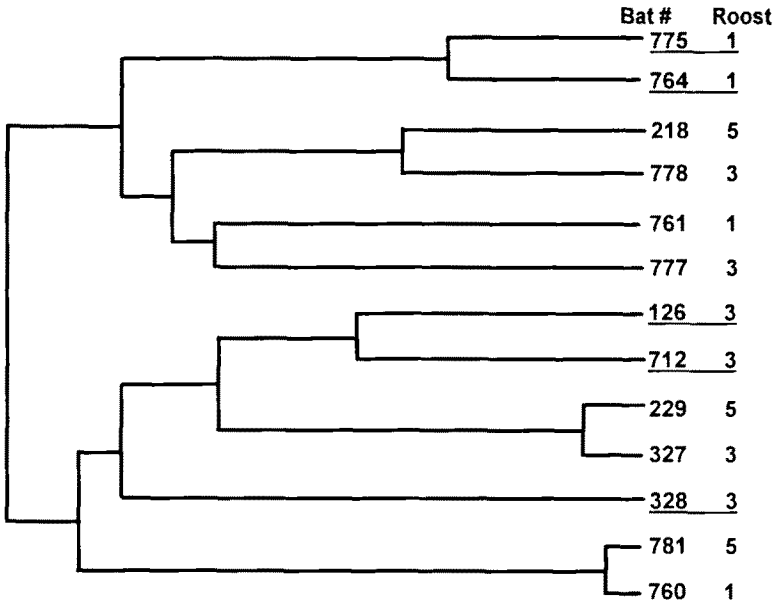

FIG. 1. UPGMA cluster analysis of the relationships among glycolipid compounds found in the subaxial secretions of 13 *Noctilio leporinus* from Culebra, Puerto Rico. Males underlined.

Although two pairs of males from the same roost grouped together, females were not grouped by roost affiliation (Figure 1). After separation of lipids during analysis, the distinctive smell of secretions was present only in glycolipids.

In the nonpolar lipid analysis, five males were more similar to each other than to females in the concentrations and numbers of compounds (Figure 2). There was no clear separation of males from different roosts. Two females from the same roost were more similar to each other than to any other individual.

The odor of harem males was very noticeable to human observers as bats foraged for insects over fields at night. On six occasions when radio-tagged male bats flew within 5 m of our position, the musky scent was obvious. Arrivals and departures of resident males at the night roost were also evident from odor alone ($N = 7$). Conversely, females were not identifiable by their odor when foraging close to our position ($N = 4$) or when flying within the night roost ($N = 9$).

DISCUSSION

Subaxial secretions were a mixture of glycolipids, nonpolar lipids, and phospholipids that varied among individuals in composition, concentration, and

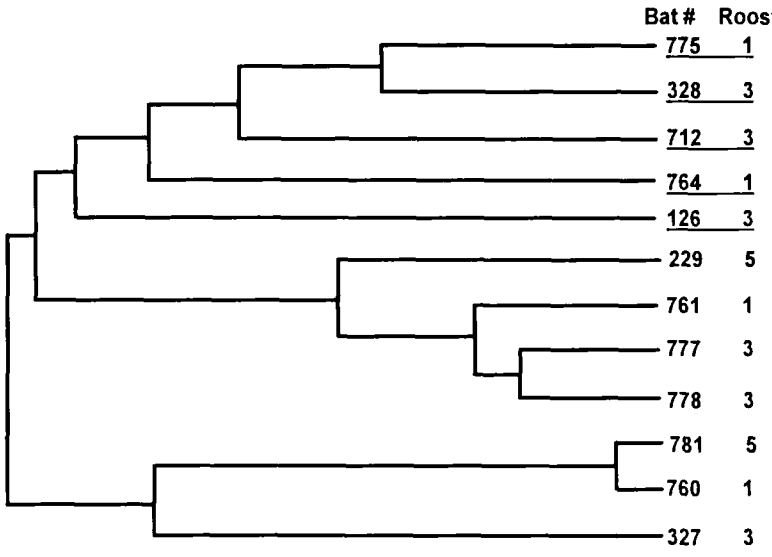

FIG. 2. UPGMA cluster analysis of the relationships among nonpolar lipid compounds found in the subaxial secretions of 12 *Noctilio leporinus* from Culebra, Puerto Rico. Males underlined.

the number of compounds. A number of compounds were present only in males or females, suggesting that scents may be sex-specific (Tables 1 and 2). Further evidence of dimorphic scent comes from the similarity of nonpolar lipid compounds in the five males. Production of chemicals in scent glands has been linked with an individual's hormonal status (Ebling, 1977). As with secondary sexual characteristics of other mammals, the strong scent of males' subaxial secretions may advertise social status.

The individually unique chemical cocktail of subaxial secretions suggests that odor profiles differ among *N. leporinus*. Differences in type and quantity of chemicals in subaxial secretions may form the basis for individual recognition by olfaction. Individual *N. leporinus* smelled the head and wings of conspecifics when they first met, both in day and night roosts. This interest in individual smell suggests that scent plays a role in individual identification. Whether *N. leporinus* is able to distinguish among conspecifics by scent was not tested in this study and remains to be answered.

The smaller amount of subaxial secretions in most females compared to males may be a result of behavioral removal of secretions by head rubs. When female *N. leporinus* rub their heads together, secretions are probably transferred between individuals. Through this scent transfer, the mixture of individual scents may develop a group olfactory identity.

The similarity in both concentration and specificity of glycolipids between males from the same roost suggests that these individuals may develop comparable odor profiles, possibly as a result of frequent interactions. The presence of nonpolar lipids in males distinguished them from females but showed no apparent within-roost or within-group distinctiveness. As phospholipids are both odorless and tasteless to humans and, presumably, to other mammals, they may not contribute to a communicative scent. Consequently, lack of differences among bats of different sex and roost affiliation is not surprising.

Mammalian scent-marking behavior is recognized to play a role in signaling social status (Brown, 1979). Many terrestrial mammals, such as hyenas, badgers, and wolves, regularly mark territorial boundaries with chemical signals (Macdonald, 1985). Female little brown bats may mark trees, possibly to aid orientation between foraging areas and roost when young are first learning to fly (Buchler, 1980). *Noctilio leporinus* were not observed using subaxial secretions to scent mark; however, the strong odor of harem males is broadcast when the bat flies. Odor trails left by these males may serve to advertise foraging territories and arrivals at night roosts. Schnitzler et al. (1994) reported that a male *N. leporinus* they watched defending a foraging territory from other bats was accompanied by a strong wave of odor. Similarly, the sweet odor of two other neotropical bat species, *Tonatia bidens* and *Mimon crenulatum*, may also function in social behavior, as the males' secretions can be detected as a bat flies nearby (Brooke, personal observation).

If male *N. leporinus* use odor trails to advertise their presence, other individuals might be able to detect small amounts of the scent. The sensitivity of bats to intraspecific odors is unknown, but the sensitivity of some bats to fruit odors has been examined. The short-tailed fruit bat (*Carollia perspicillata*) is able to locate fruit exclusively by olfaction (Laska and Schmidt, 1986). When tested for sensitivity to fruit odors (esters, alcohols, and carbon acids), short-tailed fruit bats were able to detect from 3.6×10^{15} to 2.7×10^{10} molecules/cm^3 (Laska, 1990). In comparison, moths that orient toward the source of an airborne chemical plume can detect 10^3–10^6 molecules/cm^3 (Dusenbery, 1992). Bats may be able to detect airborne odor trails, but the morphological variation in the number of olfactory receptor cells and amount of olfactory muscosa suggest that bat species differ greatly in olfactory ability (Schmidt, 1985).

We have described the chemical constituents of subaxial secretions, but how the the secretions are important to *N. leporinus* remains unknown. Do bats distinguish the odors of other individuals? Do female groups have a distinct group odor? Future experimental work should be considered with captive populations where diet can be both controlled and manipulated, reproductive status monitored, and subaxial secretions sampled repeatedly from the same individual over time.

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ATTRACTANT PHEROMONE FOR MALE RICE BUG, *Leptocorisa chinensis*: SEMIOCHEMICALS PRODUCED BY BOTH MALE AND FEMALE

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Abstract—GC profiles of the airborne volatiles produced by adult males and females of the rice bug, *L. chinensis* showed no qualitative chemical dimorphism. However, GC-EAD experiments showed that eight of the compounds elicited strong responses in male and female antennae. Ruling out alarm pheromone and compounds that were not found in the whole-body extracts of the bugs, four compounds remained to be tested as possible attractants. In field experiments, the whole mixture or tertiary blends were not attractive; however, males were strongly attracted to a 5:1 mixture of 2-(*E*)-octenyl acetate and octanol. The attractancy of the binary mixture was decreased by the addition of 3-(*Z*)-octenyl actetate. Although the binary lure specifically attracted males, there was no evidence that it triggered any response of sexual behavior in males.

Key Words—Heteroptera, Alydidae, GC-EAD, 2-(*E*)-octenyl acetate, octanol, 2-(*E*)-octenal, 2-(*E*)-octenol, octyl acetate, 3-(*Z*)-octenyl acetate, rice bug, *Leptocorisa chinensis*

INTRODUCTION

There are some 35,000 species of Heteroptera worldwide, yet sexual pheromones are fully known only for a few species. The diverse nature of the group

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itself has hindered progress, and the copious defensive secretions from metathoracic glands found in most true bugs can contaminate sex pheromone samples (Aldrich, 1995). In order to alleviate the problem derived from their overwhelming chemical fortification, we have used gas chromatography coupled with an electroantennographic detector (GC-EAD) to study chemical communication in the bean bug, *Riptortus clavatus*. This technique allowed us not only to separate defensive substances from semiochemicals but, more importantly, to isolate an alarm pheromone (Leal and Kadosawa, 1992) from other attractants. Further studies led to the identification of male-specific multifunctional semiochemicals that play the double role of attractant pheromone for adults as well as aggregation pheromone that enables second instars to find the host food plant (Leal et al., 1995). This prompted us to investigate chemical communication of other species of the same family (Alydidae). In that connection, the rice bug, *Leptocorisa chinensis* (Dallas), *kumoherikamemushi* in Japanese, is an interesting case study because it is an economically important pest in Japan, China, and other South East Asian countries for which alternative methods of control are badly neeeded. Surprisingly, we found that males and females of the rice bug produce various semiochemicals that are sensed by both sexes, two of them being attractive only to males.

METHODS AND MATERIALS

Analytical Procedures. Gas chromatography (GC) was carried out with a Hewlett-Packard 5890 II Plus instrument equipped with a split/splitless injector, electronic pressure control, a flame ionization detector, and an HP 3365 Series II Chemstation. High-resolution GC analyses were performed with polar and nonpolar capillary columns: HP-Innowax and HP-5MS (30 m × 0.25 mm × 0.25 μm; Hewlett-Packard), respectively. These columns were operated at 70°C for 1 min, increased to 230°C at a rate of 10°C/min, and held at this temperature for 10 min. Low-resolution mass spectrometry (MS) was carried out with an HP 5890 II Plus gas chromatograph linked to a mass selective detector MSD 5972 (Hewlett-Packard). Chromatographic resolution was achieved on a HP-Innowax column operated under the same conditions as described above. MS were also obtained on an Hewlett-Packard gas chromatograph electron ionization detector GCD Series, equipped with an HP-5MS capillary column that was operated at 50°C for 1 min, increased to 150°C at a rate of 5°C/min, held at this temperature for 1 min, increased to 230°C at a rate of 10°C/min, and finally held at this temperature for 10 min.

Gas Chromatography with Electroantennographic Detector (GC-EAD). The responses of *L. chinensis* antennae were recorded with a GC-EAD system (Struble and Arn, 1984). An HP 5890 Series II Plus Gas chromatograph was modified

to have the effluent from the capillary column split between EAD and FID (3:1 ratio). Bug antennae were placed in a previously described acrylic stage (Leal et al., 1992) which was set inside the glass transfer line (2 cm away from the GC outlet) and connected with gold wires to an amplifier (gain 5). The signal was filtered through a passive filter (cutoff frequency 0.12 Hz) and fed into an A/D 35900E interface (Hewlett-Packard). Simultaneous acquisitions of FID and EAD signals were obtained with the above chemstation.

Extracts. For the collection of airborne volatiles, field-collected adult bugs, separated into male and female groups, were anesthetized with CO_2 to minimize the release of defensive secretion and transferred to an all-glass aeration apparatus. The aeration apparatus contained a piece of wet cotton and an ear of foxtailgrass, *Setaria viridis* (Gramineae). The chamber was placed inside an incubator maintained at 25°C and a 16L:8D photoperiod. Volatile compounds were trapped on a Super Q (Alltech, Deerfield, Illinois) column. The trapped compounds were eluted by washing the column with hexane and the extract was concentrated to 0.1 insect equivalent/μl. Whole-body extracts were obtained by immersing at least three individuals in hexane for 3 min.

Chemicals. 2-(*E*)-Octenyl acetate and 3-(*Z*)-octenyl acetate were prepared by the reaction of the respective alcohols with acetyl chloride in dichloromethane. Octyl acetate, 2-(*E*)-octenol, and octanol were purchased from Tokyo Chemical Industry Co. Ltd.

Traps and Field Tests. Newly developed sticky traps (20 × 20 cm) were made of waterproof paper coated on both sides with adhesive polybutadiene. Field experiments were conducted in a rice field inside Ibaraki Research Center in the summer of 1995. Chemicals (1 mg) were incorporated in plastic pellets (4–5 mm in diameter) made of polyethylene-vinyl acetate. Ten pellets were loaded on the sticky plate traps, and tests were run with the traps distributed around a rice field with three replicates. Traps were placed at 25 cm above the ground with an intertrap distance of 10 m. Capture data were collected daily for at least three consecutive days; these data were transformed to log $(x+1)$ before difference among means were tested for significance by ANOVA with the JMP Software, Version 2 (Anonymous, 1989). Treatments followed by the same letters are not significantly different at the 5% level in the Tukey-Kramer honestly significant difference test.

RESULTS AND DISCUSSION

The GC-EAD profile of the airborne volatiles collected from *L. chinensis* females showed the occurrence of at least eight peaks that were EAD-active (Figure 1). The same EAD responses were obtained with male or female antennae as the sensing element.

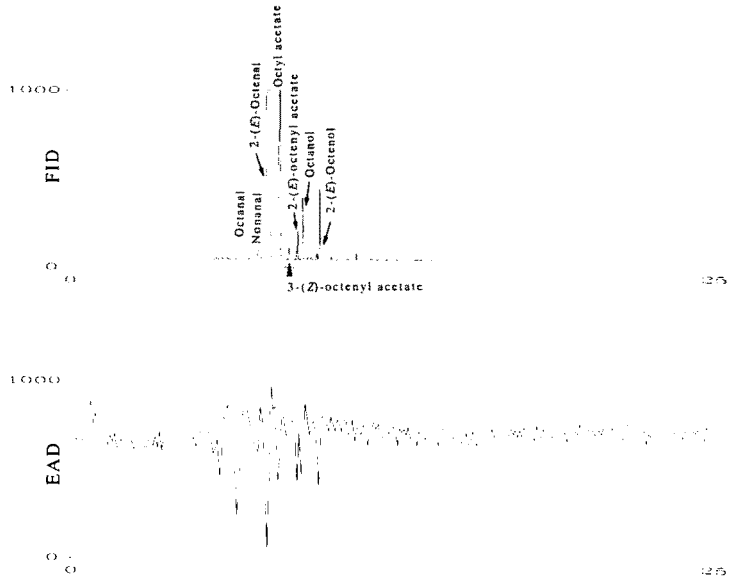

FIG. 1. Parallel FID and EAD chromatograms obtained from airborne volatile of *L. chinensis* females during the scotophase (0.1 female equivalent) and separated on an HP-Innowax column. The EAD profile using a female antenna as the sensing element (in the figure) was identical to the profile obtained with male antenna (not shown).

Interestingly, these EAD-active peaks were detected in the extracts from males as well as from females not only in the scotophase (Figure 2), but also in the photophase. The FID profiles showed only quantitative differences. There was no qualitative difference in the whole-body extracts of males and females.

The EAD-active peaks were identified by their mass spectra, followed by comparison with authentic compounds that gave the same retention times as the natural products on both polar and nonpolar columns. Because the bugs released abundant amounts of 2-(*E*)-octenal when disturbed, this compound was considered to be part of the alarm pheromone system. In fact, 2-(*E*)-octenal has been previously identified as an alarm pheromone of other hemipterans (Blum, 1985). Toxicity and repellency tests also showed that 2-(*E*)-octenal is part of the defensive secretion of the rice bug, *L. oratorius* Fabricius (Gunawardena and Bandumathie, 1993). Octanal and nonanal were ruled out as part of an attractant pheromone since these compounds were not found in the whole-body extracts.

Four compounds remained to be tested as possible attractants for the adult bugs: octyl acetate (OAc), 2-(*E*)-octenyl acetate (2EOAc), octanol (OL), and

FIG. 2. GC profiles of the airborne volatiles of (A) female and (B) male *L. chinensis* collected during 6 hr of the scotophase.

2-(*E*)-octenol (2EOL). The activity of these compounds was tested in the field by utilizing the ratio typically found in the airborne extracts; the ratio of 2EOAc, OAc, 2EOL, and OL was 10:5:2:2. This is not the ratio of the sample shown in Figure 1. Preliminary experiments showed that captures in traps baited either with a full mixture or with tertiary mixtures were not significantly different from catches in control traps.

Finally, the attractants were identified by testing binary combinations of the four compounds. These experiments revealed that a mixture of 2EOAc and OL was very attractive to males (Figure 3).

Catches in traps baited with 2EOAc and 2EOL, albeit small, were also significantly higher than captures in control traps. Further experiments involving only two treatments confirmed that males were attracted significantly more to the 2EOAc + OL lure than to the 2EOAc + 2EOL; the latter also captured significantly more bugs than control traps (Figure 4). The results were clear-cut because there was no catches at all in control traps throughout these experiments. In the preliminary tests, however, bugs were captured in control traps, probably due to the effect of weeds in the vicinity of the traps. This interference was completely eliminated by cleaning the rice field before the next series of experiments.

It was found that both male and female *L. chinensis* also produce 3-(*Z*)-octenyl acetate (3ZOAc), although EAD response to this compound was very

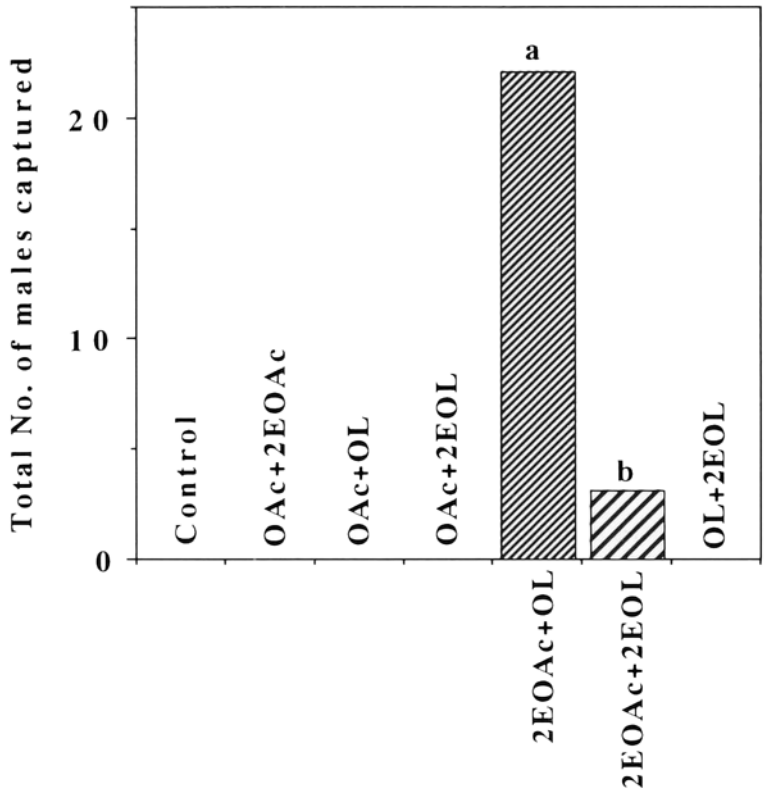

FIG. 3. Catches of *L. chinensis* males in sticky traps baited with binary combinations of the four possible attractants.

weak. Field tests showed that this compound decreased the captures of male rice bug in traps baited with the attractant mixture, 2EOAc + OL (Figure 5). Whether 3-(*Z*)-octenyl acetate is a part of the alarm pheromone system of the rice bug or a behavioral antagonist is yet to be clarified. Furthermore, 2EOL seems to have negative effects on the catches, but further experiments must be carried out to determine the role of this semiochemical in the chemical communication of the rice bug.

Sex pheromones have traditionally been considered by researchers to be those compounds that are emitted by individuals of one sex and that cause attraction of members of the opposite sex, resulting in the location of the emitter, and subsequently, mating (Baker, 1989). Contrary to many other families, sex pheromones of hemipterans are largely produced by males. It is worth noting that in alydids, the metathoracic scent glands are sometimes sexually dimorphic,

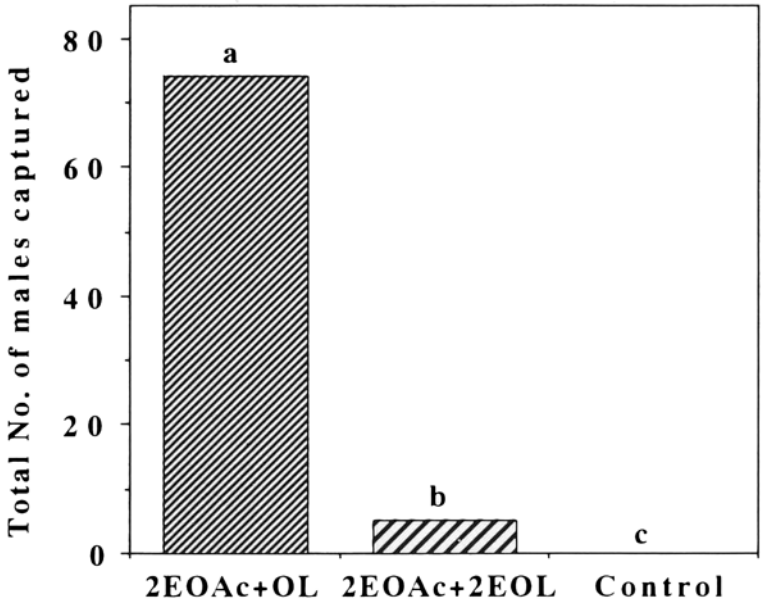

FIG. 4. Captures of males of the rice bug in sticky traps baited with 2-(*E*)-octenyl acetate plus octanol and 2-(*E*)-octenyl acetate plus 2-(*E*)-octenol.

and the dimorphisms are expressed chemically (Aldrich et al., 1993). Recently, we found that males of the bean bug *Riptortus clavatus* release semiochemicals that attract both males and females (Leal et al., 1995). Interestingly, the case of the rice bug is just the opposite, i.e., only males are attracted to semiochemicals that are produced and released by both males and females. Although female antennae of *L. chinensis* can "smell" the male attractants (and the other semiochemicals tested), they do not elicit any attraction for females. Throughout the whole series of experiments, only four females were captured, although insect samples collected in the same field had a sex ratio nearly 1 : 1.

In conclusion, 2EOAc and OL are produced by both male and female of *L. chinensis* and attract only males of the same species. Field experiments showed that a 5 : 1 mixture is very attractive, but the optimum ratio of the attractants is yet to be determined.

For this attractant pheromone we avoided using the term sex pheromone (Tamaki, 1985) because, despite the fact that the chemicals are "secreted by an organism to the outside and cause a specific reaction in a receiving organism of the same species" (Nordlund, 1981), there is no evidence that these semiochemicals trigger any sexual behavior in males other than attraction. Future

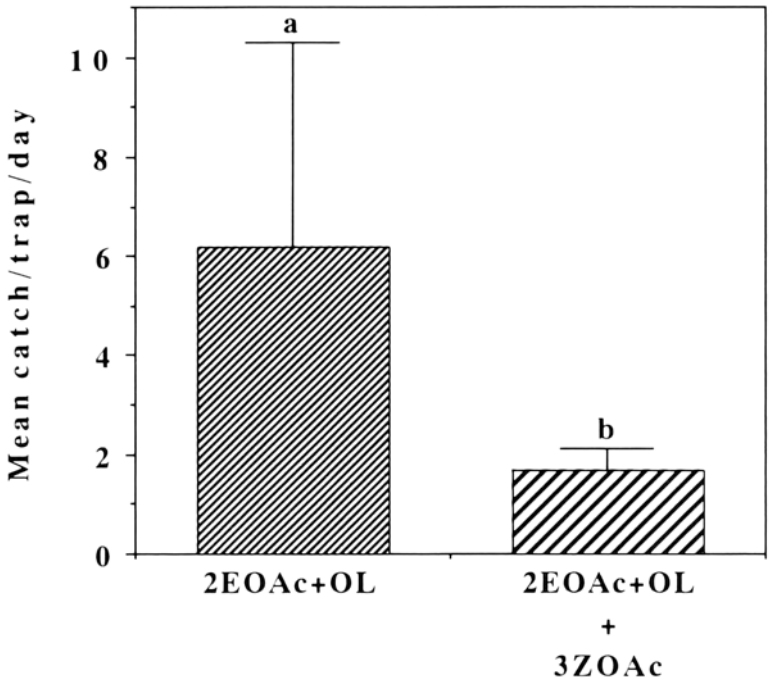

FIG. 5. Captures in traps baited with a 2-(*E*)-octenyl acetate + octanol (5 : 1) and the binary mixture plus 3-(*Z*)-octenyl acetate (5 : 1 : 1).

detailed behavioral studies will be carried out to test the possibility that these semiochemicals are involved in lek formation.

Throughout this paper we used the term semiochemicals (Nordlund and Lewis, 1976) for all EAD-active compounds because the compounds are released and sensed by the same species, and they are likely to be involved in chemical interactions between the rice bugs. Further studies are needed to clarify their specific role in the chemical ecology of this species.

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INTERSPECIFIC ADVANTAGE RESULTS IN INTRASPECIFIC DISADVANTAGE: CHEMICAL PROTECTION VERSUS CANNIBALISM IN *Utetheisa ornatrix* (LEPIDOPTERA: ARCTIIDAE)

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Abstract—This study suggests that alkaloid deficiency in *Utetheisa* (Lepidoptera: Arctiidae) is a main cause of cannibalism; moreover, cannibalism can be predicted on the basis of alkaloid deficiency and of systemic alkaloid accumulation. This chemical plays a central role in the life of this species, because, first, it provides acquired chemical protection from potential predators, and, second, it determines mating success (as the alkaloid is an essential precursor of the male pheromone). Consequently, losers in the larval sequestering of alkaloids, which would result in a lack of chemical protection and in decreased mating success, tend to target conspecific winners, which are normally substantially protected against a variety of predators; by cannibalizing those accumulated alkaloid sources the losers tend to become the winners of cannibalistic encounters while making up their shortfall of these chemicals. What is a presumptive advantage in selection under high predation pressures and/or high alkaloid availabilities could become a disadvantage under high conspecific population densities and shortages of alkaloid supplies for larval uptake. Cannibalism may be expected to have general ecological importance in the evolutionary play of *Utetheisa* and may contribute to a balanced regulation of the acquired alkaloid contents in these arctiid populations.

Key Words—Larval cannibalism, pyrrolizidine alkaloid, chemical protection, population density, *Utetheisa ornatrix*.

INTRODUCTION

Larval cannibalism is a well-known major mortality factor and for that reason could be a basic regulatory factor of populations in some species (Stinner et al.,

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1977; reviews by Fox, 1975a; Polis, 1981 and references therein). In such a function cannibalism could lower population densities to levels where resource limitation is merely a minor cause of mortality (e.g., Polis, 1980). Other factors promoting cannibalism were identified, such as food availability or quality (e.g., Banerjee and Mahapatra, 1987), developmental stages (e.g., Polis, 1981), and physical conditions including parasitization (Dindo, 1990). However, most reports about cannibalism are anecdotal and are often passing remarks within other studies dealing with problems of laboratory cultures and/or of parameters such as size, age, molting stages and/or gender.

Larvae of *Utetheisa ornatrix* (Lepidoptera: Arctiidae), the subjects of this study, feed on plants of the genus *Crotalaria* (family Leguminosae), long known to contain pyrrolizidine alkaloids (PAs). The larvae preferentially eat the seeds that have the highest concentrations of the alkaloids within the plant (Eisner and Meinwald, 1987 and references therein). Unlike most other larvae, *Utetheisa* tolerate these alkaloids. Moreover, they sequester those chemicals systemically and retain them through metamorphosis into the adult stage. However, the retained alkaloid supply may vary drastically in adult moths depending on larval access to *Crotalaria* seeds. Individual moths reared outdoors on seed-bearing *Crotalaria spectabilis* contained 701 $\pm$ 59 (mean $\pm$ SEM) μg monocrotaline, while the ones reared on immature (seedless) *Crotalaria spectabilis* contained only 98 $\pm$ 10 μg (Conner *et al.*, 1990). Natural variation and the sometimes substantially reduced quantities of PAs in *Utetheisa* could result from short supplies of *Crotalaria* seeds, competition within high population densities of *Utetheisa* for available seeds, interspecific competition with *Etiella zinckenella*, a pyralid moth that is also able to feed on that plant, or from combinations of these. A sufficient systemic chemical load is essential for chemical protection and also essential in synthesizing the male pheromone, a significant attractant for successful mating (Eisner and Meinwald, 1987).

One might imagine that *Utetheisa* larvae are unlikely to become cannibals due to their chemical protection by alkaloids (Eisner and Meinwald, 1987). However, larval *Utetheisa* that are deficient in alkaloids could make up their shortfall by turning to pupal or egg cannibalism (Bogner and Eisner, 1991, 1992). This specific alkaloid-biased cannibalism was shown to be primarily a consequence of an alkaloid deficiency rather than hunger. Specifically, it was shown that larvae acquire alkaloids by feeding on conspecific pupae or eggs that are not deficient in this alkaloid. However, this kind of cannibalism is either rare, because larvae normally pupate away from their food plants, or inefficient, in terms of alkaloid uptake. For example, the alkaloid contents of eggs are at microgram levels, magnitudes lower than necessary for successful use. In that context, larval cannibalism would be more likely in nature and could be a sufficient way of making up alkaloid. Therefore, a series of larval cannibalism experiments was undertaken in order to determine if larvae are predisposed to

be cannibalistic when they are PA-deficient and to test an hypothesized existence of chemically biased conspecific foraging, i.e., if larvae display a high preference for PA-containing larvae when given a choice.

METHODS AND MATERIALS

Experimental Animals

The *Utetheisa* were from a laboratory culture, established with stock taken near Lake Placid, Highlands County, Florida. Voucher specimens are on deposit (Lot 1154) in the Cornell insect collection. The moths were reared on two diets: (1) PB diet, a semisynthetic diet (c.f. Miller et al., 1976) based on pinto beans and devoid of PAs (shown by a minus sign in the following text), and (2) CS diet, identical to PB diet, but with 10% (by weight) of its pinto bean content replaced by ripe *Crotalaria spectabilis* seeds (shown by a plus sign). *Crotalaria spectabilis*, one of the principal foodplants of *Utetheisa* in the southeastern United States, contains the PA, monocrotaline, mostly in its seeds (Johnson et al., 1985). It is known that the CS diet is an appropriate substitute for the natural foodplant. Individuals raised on that diet contain 782 $\pm$ 141 (SEM) μg monocrotaline/moth (N = 4) (J. Kopecky, cited in Bogner and Eisner, 1992).

Basic Experimental Protocol

For the experiments, the caterpillars were transferred directly from the laboratory colony (no starvation times) to test containers (85 mm diameter, 35 mm height) with a bottom filter paper (diameter = 75 mm). The containers provided no food, but a small wet cotton ball supplied humidity. For the singular tests, first, third, or fifth instars (see below) were placed in the containers kept at a constant 23°C for the duration of the experiment time in a temperature-controlled room with 16L:8D photoperiod.

Utetheisa caterpillars show no external genitalic sex differences. However, randomly dissected samples showed equal distributions of sexes. Each larva, taken at least one day after the last molt, participated in only one test. First counts of survivors were made after 24 hr, followed by further successive 24-hr counts. No replacements were made for cannibalized larvae. It was not possible for the larvae to crawl out of the container during the experiment. In the case of the fifth instars, the tests were interrupted when the first pupation within a cage took place, which is the reason for lower numbers in fourth experimental day in test series 2 and 3.

The design of the experiments was aimed at unveiling any type of larval cannibalism in *Utetheisa*. Population densities or different body sizes were examined to look for potential clues of cannibalism (Semlitsch and West, 1988). Alkaloid dependency was hypothesized as the main cause of cannibalism.

Experimental Rationale

Test Series 1: Varying Density Parameters. The first experimental series was designed to evaluate potential density and/or alkaloid dependencies in larval cannibalism of *Utetheisa*. Roughly same-sized third instars of the same intermolting periods (middle size, 12–15 mm) were used. The experiment involved placing 5, 10, 15, or 20 larvae per cage, and included 15 replicates within each density. Only (+)-alkaloid or (−)-alkaloid-grown were grouped together in each test series. In each case the survival rate was scored every 24 hr during the following seven days.

Test Series 2: Size Dependency (Control Design for Test Series 3). Similar to the experimental design of the previous test series, the second experiment focused on potential cannibalism caused by differential larval body size and was a control for the later test series 3. Sets of second instars (small-size, 6–8 mm) or fifth instars (large size, 24–27 mm) were caged, and survival rates were monitored during three consecutive starvation days. The experimental time was limited by pupation after four days of the large caterpillars. In each test, 10 same-sized caterpillars, either (+)- or (−)-grown, shared containers. Each test was replicated 15 times.

Test Series 3: Asymmetric Encounters of Different PA-Content. In the third experiment body size and alkaloid content were brought into the design as additional parameters. Although the experimental larvae were not labelled according to their alkaloid content, due to the different body sizes, the alkaloid content of the subjects could be assured: (+)- and (−)-grown larvae could be distinguished by body sizes only when they had been placed together in a common cage. In each case, 10 caterpillars, for instance, five large (+) and five small (−) etc., shared containers during four sequential starvation days, covering all combinations of size (large/small) and alkaloid content (-laden/-deficient). The purpose of this test was to disentangle size and alkaloid dependencies in asymmetric-sized conspecific clashes. Each test was replicated 25 times.

Field Observation

The study sites for the field observation were patches of *Crotalaria mucronata* at the Archbold Biological Station or at the Agro-Ecology Research Station, both in Highlands County, Florida. The plants were of all ages, many of them mature and with seed pods at all developmental stages, but they were only moderately infested with *Utetheisa*. During field observation periods, this low population density was indicated by only a few larvae per plant, although intact seed pods were in ample supply. However, many larvae could be present within the seeds and therefore not (or only barely) visible in heavy infestations and consequently dozens of *Utetheisa* larvae may be present on single plants (Eisner,

personal communication). Most of the plants were inspected at irregular intervals.

Statistical Protocol

For testing the significance of the analysis of variance within the density experiments (Figure 1), a MANOVA test of between-subjects affects was undertaken. In Table 1 statistical differences of the linear effect of day 0 to day 8 are presented. Repeated measures using orthogonal polynomial transformations were performed. Only the linear trend differed significantly from zero when tested by a classical t test. However, since the typical data for this test are raw data values, here the differences between values are tested. In the case of the cross-cannibalism experiments (Figure 3 below), for the between-group comparisons, median comparisons were performed using Mann-Whitney U-Wilcoxon's rank sum W test by computing a linear trend calculation (Table 2). Details of these

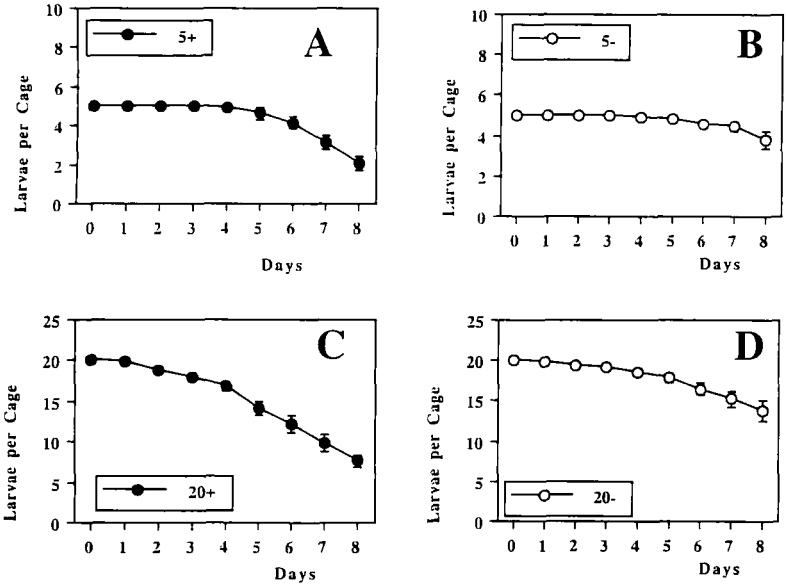

FIG. 1. Survival rate of same-sized third-instar *Utetheisa* caterpillars caged in numbers of 5 and 20 per container: one design with (+), the other with (−) specimens [(−) means alkaloid-deficient, (+) alkaloid-laden]. The results of identical experiments with 10 and 15 caterpillars per cage are displayed in Table 1. No food was presented during the eight-day periods (values are means, bars are ± SEM). Each experiment had 15 replicates.

TABLE 1. TESTS OF LINEAR EFFECTS CONCERNING DENSITY POPULATION TESTS (DATA OF FIGURE 1)

	20+	20−	15+	15−	10+	10−	5+	5−
Mean (linear trend)	−6.81	−12.40	−5.06	−10.06	−2.13	−5.09	−1.20	−1.87
SD	5.17	2.89	4.07	3.47	2.42	2.51	1.70	1.46
SEM	1.29	0.75	1.02	0.87	0.61	0.77	0.44	0.38
Mean difference	5.59		5.00		2.97		0.67	
t-value	3.74		3.74		3.08		1.15	
df	23.88		30.00		25.00		28.00	
Two-tail significance	0.001		0.001		0.005		0.258	
SE of difference	1.49		1.34		0.96		0.58	
95% CI for difference	2.51; 8.67		2.27; 7.73		0.99; 4.95		0.52;1.85	

tests are given in Siegel (1956). All computations were performed using the statistical package SPSS for Windows (see Norusis, 1993a,b).

RESULTS

Experiment 1: Rate of Cannibalism and Density Between Caterpillars with Identical Alkaloid Content. Even high population densities caused no severe intraspecific feeding in middle-sized caterpillars after the first starvation day (Figure 1, Table 1), but, as expected, survival rates were dependent on popu-

TABLE 2. MEDIAN COMPARISON OF THE TWO VARIANTS (+) AND (−): MANN-WHITNEY U-WILCOXON'S RANK SUM W TEST (DATA OF FIGURE 3)

Differences in Figure 3	U	*N*	*p*
D			
Day 4	27.5	50	0.0001
Day 3	29.5	50	0.0001
Day 2	59	50	0.0001
Day 1	125	50	0.0001
B			
Day 4	49.5	35	0.0001
Day 3	151.5	50	0.0001
Day 2	174	50	0.0001
Day 1	250	50	0.02

lation densities. Not surprisingly, the more crowded a cage, the more cannibalism occurred. However, hunger should not depend on density since no food was provided in any of the cages, but larvae may simply feed in part on larvae dead from starvation (although this has never been observed). Nevertheless, the survival rates were high in comparison to other studies (Semlitsch and West, 1988; see Discussion), even after long starvation periods. However, the mortality was significantly dependent on the systemic PA content of the caterpillars, because death rates in alkaloid-containing larvae were twice as high, even in less crowded situations, as in alkaloid-deficient caterpillars (Figure 1B–D). However, on the basis of this experimental design alone, the possibility still exists that the mortality rates of (−) and (+) larvae differ (see below).

Experiment 2: Cannibalism Rate and Larval Body Size. Middle-sized caterpillars, caged as 10 per container, displayed almost no cannibalism within the first three days, i.e., it was identical to the situation of five caged caterpillars in Figure 1 (A and B). This was also true, given the same population densities, in the case of small-sized and large-sized larvae during the tested period. Therefore, body size did not seem to play any considerable role in cannibalism when (+) and (−) caterpillars could not encounter each other (Figure 2A–D).

Experiment 3: Cannibalism of Different-Sized Caterpillars with Different Alkaloid Content. Caged middle-sized caterpillars showed an alkaloid-dependent probability of being cannibalized when the starvation period lasted long enough (Figure 1); in these tests the encounters were of equal body size and of equal alkaloid content. The observed cannibalism rate was also low when large or small caterpillars shared the same container (Figure 2). When physical advantage was taken into account, the cannibalism rate was highest in the case where small alkaloid-laden caterpillars shared the cage with large alkaloid-deficient caterpillars (Figure 3D). The same design, but the reversed constellation (small alkaloid-deficient and large alkaloid-laden) revealed cannibalism, too, but at substantially lower rates (Figure 3C). Both controls, that is, larvae of both sizes containing equal alkaloid backgrounds also revealed lower mortality rates (Figure 3A,B). The small-sized "haves" apparently faced a considerably greater risk from large-sized "have-nots" (Figure 3D, Table 2). Large caterpillars might be better in defending themselves as well as more prone to attacking when in need of the alkaloid.

Field Observation. Cannibalism was only rarely observed in a field population of *Utetheisa*, although causal observations become relevant at this point. Caterpillars often pierce holes in the *Crotalaria* pods and crawl into the pods until they are completely hidden. During the time when the invasion takes place, the caterpillars may poorly oppose an attack while presenting a vulnerable abdomen towards the attacker. In two observed cases, the incumbent caterpillar could not turn around quickly enough within the pod (or retreat out of the gnawed pod) and could not resist the attack, and thus was cannibalized. The (successful)

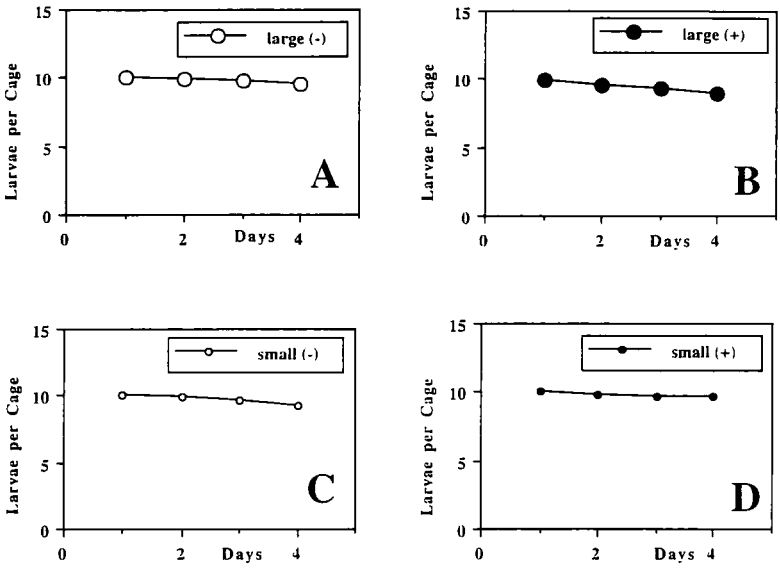

FIG. 2. Survival rate of 10 *Utetheisa* larvae per cage [large means fifth instar, small means second instar; (−) is alkaloid-deficient, (+) is alkaloid-laden]; 15 replicates were undertaken (in the case of the fourth day, due to pupation $N < 15$). Values are means (SEM not shown).

challenger ate the leaking hemolymph and cannibalized the caterpillar. Cuticular residues found within the pods may point to cannibalism, too.

DISCUSSION

The most important implication of the experiments presented is the demonstration of the likelihood that an alkaloid is the driving force for cannibalism in *Utetheisa* caterpillars. Intraspecific mortality seems to be caused basically by the deficiency and availability of the alkaloid rather than by other parameters alone, such as exaggerated population densities or starvation. Under the simple choice conditions of the present laboratory tests, *Utetheisa* larvae may opt to forage upon conspecific larvae that are alkaloid-laden. Although there is a decrease in the larval numbers due to starvation, alkaloid content is shown to be linked to susceptibility to cannibalism. The chemically protected caterpillars of *Utetheisa* are less likely to survive than their alkaloid-deficient conspecifics.

Although it is still unclear how the caterpillars identify the alkaloid within conspecifics and how they could sense which conspecifics carry alkaloids, the

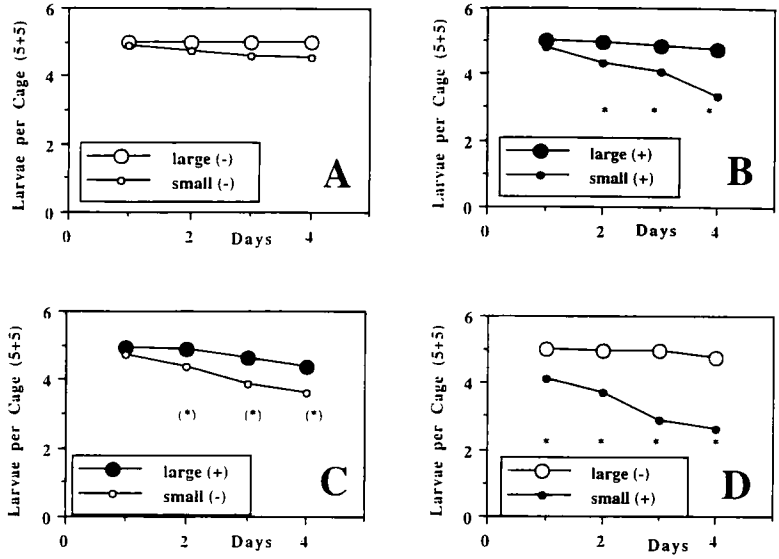

FIG. 3. Survival of five large (fifth instar) and five small (second instar) caterpillars, caged in the same containers. The design allowed recognizing of larval alkaloid contents without artificial markings. No food was presented during the experimental period. Each experiment had 25 replicates (in the case of the fourth day, due to pupation $N < 25$). Values are means; z-values: * ≤ 0.001, (*) ≤ 0.01).

experiments presented identify the systemic alkaloid content as the major cause of being cannibalized. This is in line with previous work (Bogner and Eisner, 1991, 1992). Chemical analyses indicate that in *Utetheisa* larvae, one single cannibalistic act is sufficient to provide the cannibals' drug requirement (see also, Bogner and Eisner, 1991, 1992).

The alkaloid content of *Crotalaria* provides the plant with considerable chemical protection. Among other attributes, the chemicals are extremely potent toxins in mammals (Mattson, 1980). Therefore, species such as *Utetheisa*, which can use as a food plant plants that other herbivore species avoid due to chemical load, might be less likely limited by food scarcity than other plant-eating caterpillars. Less interspecific competition towards food resources could result in less intraspecific larval competition. Laboratory experiments with *Utetheisa* reveal little cannibalism within the experimental time compared to other studies (Semlitsch and West, 1988). Although we do not know about relevancies of the latter experiment for *Utetheisa*, and although the *Utetheisa* population could peak at high densities (see Materials and Methods, Field Observations), cannibalism in *Utetheisa* could be less necessary to reduce oversized populations

before, for instance, mass starvation occurred (Fox, 1975b). However, in very high population densities, where heightened feeding activity results in more frequent confrontations, more harmful intraspecific interactions take place, resulting in higher cannibalism rates and consequently contributing to lower densities (Breden and Chippendale, 1989).

Intraspecific larval confrontation often has resulted in cannibalism, especially when encounters occurred between asymmetric sizes of larvae. Smaller conspecifics were more frequently eaten than larger ones (Polis, 1981; Semlitsch and West, 1988). This is also true in the case of *Utetheisa*, but only under certain circumstances, such as the larger counterparts being in need of alkaloid. The greatest probability of becoming cannibalized was observed when small alkaloid-laden caterpillars were encountered by large alkaloid-deficient ones. The parameter of small body size could not be the sole determinant of becoming a cannibalized victim, but the body content of alkaloid appears to be the reason for being predisposed to being a cannibal or a victim.

A persistent weakness of most studies on larval cannibalism has been the evaluation of cannibalism in natural populations. The present study does not compensate for this insufficiency. However, in the case of *Utetheisa* there may be much (invisible) cannibalism within the pods. If a caterpillar is hidden within pods and another one tries to invade the pod, a cannibalistic encounter would be almost inescapable, especially when a potential invader faces the (vulnerable) abdomen of the incumbent that has not completed its invasion into a pod. Variations of larval alkaloid content in nature could result from the host plants containing alkaloids in varying amounts, from insufficient access to the seeds (see above), or from both.

Although chemical protection by alkaloid load is only one facet of the chemical ecology of the animal, it could be argued that potential predators use the aposematic coloration of *Utetheisa* as an avoidance cue. They may not be able to assess the chemical load of individual caterpillars via volatile principles as the adult *Utetheisa* can (Bogner et al., 1992). This would bring automimicry into play since the systemic alkaloid content varies. Besides chemical protection the alkaloid also plays a central role as a basic chemical for the biosynthesis of the male pheromone, hydroxydanaidal. Just as the male transfers a spermatophore, the female transfers the alkaloid load into the eggs (Dussourd et al., 1988), providing essential protection for this vulnerable life stage. Given the importance of the alkaloids in the life of *Utetheisa*, caterpillars might cannibalize alkaloid-laden conspecifics for their own source when in need.

Beyond the fitness of *Utetheisa* caterpillars in terms of chemical protection and mating success (Eisner and Meinwald, 1987), it could be argued, although without any labeling evidence for this assertion, that the alkaloid also could be a major source of nitrogen. Such energy transfer has been discussed in related studies (Mattson, 1980; Banerjee and Mahapatra, 1987). Extended starvation

stress causes an increasing cannibalism rate in *Utetheisa*, which was substantially lower than the rate in other species (Semlitsch and West, 1988). Conspecific caterpillars are potential competitors while feeding on potentially limited food resources. Finally, in *Utetheisa*, the alkaloids function as phagostimulants, as was shown in previous studies (Bogner and Eisner, 1991, 1992). The alkaloids, therefore, could function as an essential cue for caterpillars to direct attacks preferentially on larvae that are PA-laden. This should be even more important when caterpillars can move between plants, since in such cases even more substantial differences between the caterpillars could be expected. However, the cannibalistic threat could result in behavior whereby *Utetheisa* caterpillars initially try to avoid each other and disperse as far as foodplant availabilities permit and offer alternatives.

CONCLUSION

The alkaloidal variance in field specimens could reflect not only availability and foraging of alkaloidal resources, but could also reflect a balancing of: (1) a cannibalism threat, (2) chemical protection, and/or (3) male fitness (in terms of the male pheromone production). The observed alkaloid amount gained during larval life could play a crucial rule. Winners in sequestering alkaloids are well protected against predators, whereas losers are not; therefore, the more alkaloid the better should be expected as the rule. Chemical protection could be replaced by automimicry (for less-protected larvae), and it could also increase cannibalism. Even when all *Utetheisa* advertise chemical protection by aposematic coloration, within the natural environment probably only the larval conspecifics are able to detect the truth in its favorable focusing on the winner. Therefore, given the importance of alkaloids in the life of *Utetheisa*, good chemical protection could be a good investment only when predation pressures are high and alkaloid availabilities are abundant. It could be a bad investment when population densities of *Utetheisa* are high and alkaloid availabilities are scarce. In that context, the general rule, the more sequestering the better, would be inapplicable, especially when the best available alkaloid sources might be conspecifics. In the latter situation, the loser would be in less danger of being cannibalized, whereas the winner would be the preferential target and victim of this intraspecific interaction. Within sexual selection (in terms of alkaloid sequestering and endowment), the balancing of the chemical load should, therefore, be the optimal evolutionary guideline.

Questions raised in Bogner and Eisner (1992) are still to be answered. Are there times when it is beneficial for *Utetheisa* to be alkaloid-deficient, such as when the threat from cannibalism outweighs that from predation? This could occur when foodplant seeds are scarce, population densities are high, and intra-

specifics would be the most concentrated alkaloid sources. There is still a lack of field data supporting the laboratory phenomenon that usurpation of alkaloids by cannibalism provides the balance of the chemical in *Utetheisa*. The laboratory data presented here should be a reliable basis to disentangle another thread of the complex chemical ecology network of *Utetheisa*.

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INSECT ANTIFEEDANT AND GROWTH-REGULATING ACTIVITIES OF SALANNIN AND OTHER C-SECO LIMONOIDS FROM NEEM OIL IN RELATION TO AZADIRACHTIN

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Abstract—The antifeedant and insect growth-regulating activities of salannin, nimbin, and 6-deacetylnimbin, in comparison with azadirachtin-A, have been studied against *Spodoptera litura*, *Pericallia ricini*, and *Oxya fuscovittata*. Salannin deterred feeding, delayed molt by increasing larval duration, caused larval and pupal mortalities, and decreased pupal weights in the two lepidopterans. Salannin also caused molt delays and nymphal mortalities in *Oxya fuscovittata*. The role of salannin and other compounds in conferring bioactivity, along with azadirachtin-A, to neem oil/neem seed extracts is emphasized.

Key Words—Antifeedant activity, insect growth-regulating activity, salannin, nimbin, 6-deacetylnimbin, azadirachtin-A.

INTRODUCTION

Neem oil and neem seed kernel extracts from *Azadirachta indica* A. Juss (Meliaceae) have been used in India since ancient times for the protection of plants from insect attack. The wisdom of this traditional practice was validated by the isolation, by Butterworth and Morgan (1968), of azadirachtin, which was stated to be a feeding deterrent against the desert locust at a concentration of 40 μg/liter. This remarkable observation aroused the interest of chemists and biologists all over the world during the past two decades, and as a result more than 100

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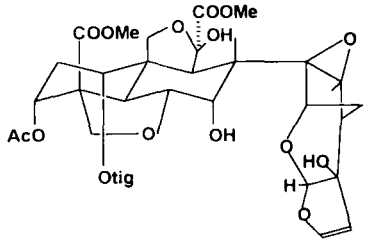
compounds have been isolated from all parts of the tree (Siddiqui et al., 1989; Devkumar and Sukh Dev, 1993; Kraus, 1995). Azadirachtin has been shown to be a potent antifeedant at 10–100 ppm and an ecdysis inhibitor at 1–10 ppm (Warthen, 1989; Govindachari, 1992; Champagne et al., 1992; Hansen et al., 1994; Schmutterer, 1995) in over 200 species of insects. Since neem seeds and neem oil are readily available, there is every promise that formulations based on them will be useful for pest control without harmful effects to the ecosystem. Indeed, a large number of formulations have been produced and marketed during the past few years, which have been standardized in terms of azadirachtin content.

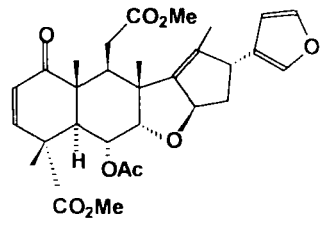
A recent study (Govindachari et al., 1995a) reported the isolation of the major triterpenoids in neem oil by preparative high-performance liquid chromatography and quantified their abundance by analytical HPLC. Salannin (1.4%), nimbin (0.5%), deacetylnimbin (0.4%), azadiradione (0.2%) and epoxyazadiradione (0.13%) are the major constituents in neem oil, while azadirachtin-A was present to the extent of only 0.03%. Interestingly, neem oil contains other azadirachtins, such as azadirachtins-B, -D, -H, and -I, which together constitute, on a conservative estimate, at least 0.2% of the oil. These azadirachtins possess the same order of activity as azadirachtin-A (Govindachari et al., 1994). Neem kernel extracts also contain all these compounds and azadirachtin-A alone is present, on average, at ca. 0.3%.

It is generally believed that the bioactivity of neem preparations is due to the azadirachtin content in them. According to Isman et al. (1990) "even though other limonoids from neem and related meliaceae have demonstrated bioactivity against insects, none of these are within two orders of magnitude as active as azadirachtin and thus their contribution to bioactivity of neem oils may be largely discounted."

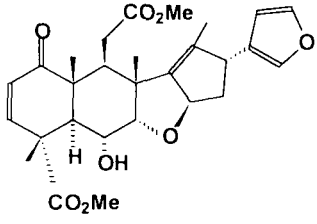
There are only a few entomological studies on the constituents of neem other than azadirachtin-A. Salannin, for instance, has been shown to be an antifeedant, as active as azadirachtin-A against *Epilachna varivestis* (Schwinger et al., 1984, Kraus et al., 1987) and more active against *Pieris brassicae* (Luo Lin-er et al., 1995). However, it had no insect growth regulatory (IGR) activity (Rembold, 1989, Simmonds et al., 1990). Azadiradione and epoxy azadiradione are feeding deterrents to a lesser extent than azadirachtin-A (Govindachari et al., 1995b). It may be noted that the major limonoids, except azadirachtin-A, have been tested for bioactivity, especially for IGR activity, only against a few insect species. While azadirachtin content in neem oil correlated well with behavior-disrupting and growth-regulating activity of *Peridroma saucia* (Isman et al., 1990), no such correlation was found with neem kernel extracts from different sources against *Epilachna varivestis* and *Aedes aegyptii* (Ermel et al., 1984). Subsequently, Ermel et al. (1987), based on 66 neem kernel extracts, claimed strong correlation of EC_{50} values of all samples with absolute amounts



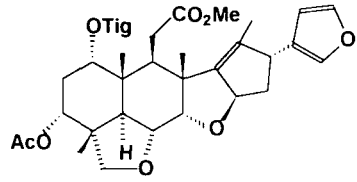
Azadirachtin A



Nimbin



6-deacetylnimbin



Salannin

FIG. 1. Limonoids tested in this study.

of azadirachtin. However, they have indicated that "because of the lack of sufficient data, this finding is statistically significant only for the Benin extract, but an obvious tendency existed with other extracts".

The present study was undertaken to assess the relative contribution of these major limonoids, viz., salannin, nimbin, deacetylnimbin, and also azadirachtin-A (Fig. 1) (for comparison) in terms of insect antifeedant and growth regulating activities.

METHODS AND MATERIALS

Salannin, deacetylnimbin, nimbin, and azadirachtin-A were isolated by a direct preparative HPLC method, their purities confirmed by analytical HPLC (Govindachari et al., 1995a), and their identities established by comparison with authentic samples (NMR and mass spectral data).

The test insects, *Spodoptera litura* and *Pericallia ricini*, were reared on the leaves of *Ricinus communis*. *Oxya fuscovitatta* was reared on the leaves of *Coix lachryma*. A dual-choice bioassay was performed (Govindachari et al., 1995b). For *S. litura* and *P. ricini*, circular disks (180 cm^2) were cut from the

leaves of *R. communis*, with the median vein as the marker between the two equal halves. One milliliter of acetone alone or the test compound in acetone (at 50, 10, 5, and 1 $\mu g/cm^2$ area of leaf) was spread with the help of a fine pipet on the right half (90 cm^2 treated), leaving the left half untreated. For each compound and concentration, five replicates were used. After air-drying, each leaf disk was placed inside separate Petri dishes and five freshly molted third instars (from the same egg batch, approximately similar in weight and size) were placed at the center of the leaf. After 24 hr, the insects were removed and the uneaten area in the treated half was measured using a ΔT area measurement meter. For *O. fuscovittata*, leaf strips (60 × 4 cm) were cut with the median vein acting as a marker between two equal halves and assayed as indicated above. Percent feeding index (PFI) was calculated (c.f. Luco et al., 1994) using the formula

$$\text{PFI} = \frac{\text{area fed in treated}}{\text{area fed in treated} + \text{area fed in solvent control}} \times 100$$

where treated is the area treated with the solution of compound in 1 ml acetone and solvent control is the area treated with 1 ml acetone.

For the insect growth regulation study, leaf disks (180 cm^2 of *R. communis* leaves for *S. litura* and *P. ricini* and 240 cm^2 of *C. lachryma* leaves for *O. fuscuvittata*) were spread with 2 ml of a solution of the test compound in acetone, to have 0.5 μg of test compound/cm^2 leaf area. Leaves treated with acetone alone were kept as control. The leaves were air-dried and placed in separate Petri dishes. Five replicates were maintained for each compound. Five freshly emerged third instars of *S. litura/P. ricini* were introduced into each Petri dish and forced to feed until they molted into the next instar. From the fourth instar, the larvae were provided with normal diet (*R. communis* leaves) until the end of the experiment. In the case of *O. fuscovittata*, five fifth instar females were introduced into the cage along with the treated *C. lachryma* leaves and the nymphs were fed till they molted to the adults. Durations of *S. litura* and *P. ricini* instars, mortality of larvae, numbers of pupae, and pupal weights were recorded. For *O. fuscovittata*, nymphal duration and mortality were recorded. The data were subjected to one-way ANOVA and Student Neumann-Keul means were presented.

RESULTS

Among the insects tested, *Oxya fuscovittata* was the most sensitive in terms of antifeedancy to all four compounds, even at concentrations of 1–10 $\mu g/cm^2$ leaf area, while *Spodoptera litura* was the least sensitive. As an antifeedant, azadirachtin was the most effective compound followed by salannin, nimbin,

TABLE 1. PERCENT FEEDING INDICES OF *Spodoptera litura*, *Pericallia ricini*, AND *Oxya fuscovittata* FED ON C-SECO LIMONOIDS FROM *Azadirachta indica*[a]

Compound	Insect species	Percent feeding index			
		1 $\mu g/cm^{2}$[b]	5 $\mu g/cm^{2}$[b]	10 $\mu g/cm^{2}$[b]	50 $\mu g/cm^{2}$[b]
Azadirachtin-A	*S. litura*[c]	27.50(4.0)	17.96(6.6)	16.50(2.8)	11.50(4.8)
	P. ricini[c]	20.80(3.5)	15.60(3.1)	12.50(3.5)	10.00(3.1)
	O. fuscovittata[d]	25.80(3.1)	18.40(3.2)	12.80(2.1)	10.60(2.7)
Salannin	*S. litura*[c]	36.50(7.5)	32.00(4.5)	30.20(5.2)	27.40(4.2)
	P. ricini[c]	29.30(5.4)	28.50(3.0)	22.50(2.2)	14.40(3.3)
	O. fuscovittata[d]	28.80(3.0)	20.50(4.8)	15.40(3.3)	12.40(2.8)
Nimbin	*S. litura*[c]	37.10(4.1)	35.50(2.0)	34.20(5.8)	26.00(3.6)
	P. ricini[c]	32.50(2.5)	30.40(3.3)	30.20(5.0)	16.60(3.0)
	O. fuscovittata[d]	26.20(4.8)	23.30(3.9)	18.20(4.0)	11.80(5.4)
6-Deacetyl nimbin	*S. litura*[c]	35.40(2.1)	35.00(3.6)	33.10(3.6)	30.50(4.4)
	P. ricini[c]	35.50(1.8)	34.00(2.6)	29.80(5.6)	20.20(3.1)
	O. fuscovittata[d]	32.40(2.6)	25.95(2.5)	20.40(1.8)	15.50(4.2)

[a]Values presented are Student-Neumann-Keul means; values in parentheses indicate ± SD.
[b]Concentration/cm^2 area of *R. communis* leaf for *S. litura* and *P. ricini*; *C. lachryma* for *O. fuscovittata*.
[c]Third instar.
[d]Fifth instar.

and deacetylnimbin in order of decreasing activity. Azadirachtin was twice as active as salannin against *S. litura* and *Pericallia ricini*; salannin, nimbin, and azadirachtin were equally active against *O. fuscovittata* (Table 1).

The insect growth-regulatory activities of the above four compounds were assessed in terms of larval duration, pupal duration, larval and pupal mortalities, and pupal weights. A significant increase in larval durations was noted in *S. litura* and *P. ricini* when fed on each of the four compounds (Table 2). The effect was pronounced in the fifth instars of *S. litura* and the third and fourth instars of *P. ricini*. No significant differences were found among salannin, nimbin, and deacetylnimbin, clearly indicating equal effectiveness in increasing the larval durations of *S. litura* and *P. ricini*. A marginal, but significant, increase in pupal duration was noted in *P. ricini* when fed on all four limonoids, while no such change was noticed with *S. litura*. In the case of *O. fuscovittata*, azadirachtin and salannin significantly increased duration of the fifth instars (Table 3).

Larval mortality was comparatively higher in azadirachtin-fed *S. litura* and *P. ricini*, while pupal mortality was as high in salannin- and nimbin-fed individuals as in azadirachtin-fed individuals (Table 2). In the case of *O. fuscovit-*

TABLE 2. DURATION OF DEVELOPMENT, PUPAL MORTALITY, AND PUPAL WEIGHT OF *Spodoptera litura* and *Pericallia ricini* FED ON C-SECO LIMONOIDS FROM *Azadirachta indica*[a]

Insect species	Compound	Duration of individual instars (days)					Mortality (%)		Pupal weight (mg)
		3	4	5	6	pupal dur.	L	P	
Spodoptera litura	Control	3.46 ± 0.5a	3.60 ± 0.45a	4.50 ± 0.82a	3.36 ± 0.78a	8.31 ± 0.46a	1.0	0	438.0 ± 10.6a
	Azadirachtin A	4.20 ± 0.86b	4.50 ± 0.62b	6.8 ± 0.85b	3.80 ± 0.41b	8.45 ± 0.52a	23.8	16	318.2 ± 12.5b
	Salannin	4.10 ± 0.35b	4.50 ± 0.5b	6.50 ± 0.5bd	3.90 ± 0.5b	8.40 ± 0.3a	13.6	12	360.0 ± 13.3c
	Nimbin	3.90 ± 0.46b	4.20 ± 0.68b	6.30 ± 0.4cd	3.50 ± 0.3a	8.50 ± 0.65a	9.1	12	385.4 ± 10.2d
	6-deacetyl nimbin	3.90 ± 0.55b	4.20 ± 0.51b	6.00 ± 0.6c	3.45 ± 0.4a	8.40 ± 0.55a	8.7	8	382.4 ± 9.5d
Pericallia ricini	Control	4.50 ± 0.70a	7.50 ± 0.2a	4.50 ± 0.35a		6.90 ± 0.38a	0	4	476.4 ± 10.5a
	Azadirachtin A	7.00 ± 0.85b	8.60 ± 0.6bc	5.70 ± 0.65c		7.50 ± 0.30b	28.5	16	344.0 ± 15.5b
	Salannin	6.80 ± 0.9b	8.50 ± 0.4c	5.20 ± 0.5b		7.40 ± 0.45b	14.3	16	381.0 ± 9.9c
	Nimbin	6.80 ± 0.6b	8.80 ± 0.5b	5.50 ± 0.6bc		7.10 ± 0.8a	18.2	12	398.8 ± 12.5d
	6-deacetyl nimbin	6.90 ± 0.72b	8.50 ± 0.5c	5.30 ± 0.35		7.00 ± 0.8a	9.5	16	412.3 ± 14.3e

[a]Values presented are Student-Neumann-Keul means; values in parentheses indicate ± SD. Experiment initiated with 25 individuals (five replicates with five individuals each). Concentration of compounds used = 0.5 $\mu g/cm^2$ area of *R. communis* leaf. Values followed by the same letter are statistically not significant at $P < 0.05$. L = larvae; P = pupae.

TABLE 3. DEVELOPMENTAL DURATION OF FINAL INSTAR OF *Oxya fuscovittata* FED ON SOME C-SECO LIMONOIDS[a]

Compound	Duration of development (days)	Mortality (%)
Control	6.2(0.55)a	1
Azadirachtin-A	8.4(0.46)b	20
Salannin	8.0(0.25)c	16.6
Nimbin	7.8(0.42)c	6.6
6-deacetyl nimbin	7.9(0.50)c	6.6

[a]Experiment initiated with 30 individuals (six replicates with five individuals each). Values presented are Student-Newmann-Keul means; values in parentheses indicate ± SD; concentration of compounds used = 0.5 $\mu g/cm^2$ area of *Coix lachryma* leaf.

tata, fifth instar mortality was much higher in azadirachtin- and salannin-fed individuals as compared to that observed for nimbin- and deacetylnimbin-fed individuals (Table 3). Significant reduction of pupal weight was characteristic of azadirachtin-fed individuals. Salannin, although less effective, brought about significant pupal weight reductions compared to nimbin, deacetyl nimbin, and the control.

DISCUSSION

In our bioassay experiments salannin, nimbin, and deacetylnimbin were half as active as azadirachtin as antifeedants. However, in growth-regulatory activity against *S. litura*, *P. ricini*, and *O. fuscovittata*, salannin is comparable to azadirachtin-A. Thus, salannin deters feeding, delays molt by increasing larval duration, causes larval and pupal mortality, decreases pupal weights in *S. litura* and *P. ricini*, and causes molt delay and nymphal mortalities in *O. fuscovitatta*. It was reported that salannin had no growth regulatory activity against *E. varivestis* (Rembold, 1989), *Spodoptera frugiperda*, *Spodoptera littoralis* (Simmonds et al., 1990), and *Heliothis virescens* (Simmonds et al., 1990, Klocke, 1987). In the present communication, salannin has been shown to be an effective insect growth regulator against some insects. Therefore, the absence of IGR activity of salannin against certain insects cannot be generalized for all insect species. In our bioassay experiments, nimbin and deacetylnimbin do not show any appreciable growth-regulating activity against *S. litura*, *P. ricini*, and *O. fuscovittata*.

Salannin, which has good antifeedant and growth-regulating activity, is present in neem oil in at least three to four times the concentration of azadi-

rachtin. Nimbin, deacetylnimbin, azadiradione, and epoxy azadiradione, although less active than azadirachtin, are also present in considerable quantities (Govindachari et al., 1995a). Therefore, while azadirachtin is undoubtedly a remarkable antifeedant and growth-regulating compound, the role of other limonoids (especially salannin) in the bioactivity of formulations should not be ignored or underestimated. Further experiments are in progress for the study of antifeedant and growth-regulating activities of salannin and other major terpenoids in neem against other insect pests.

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THRESHOLD CHEMOSENSITIVITY AND HYPOTHETICAL CHEMORECEPTOR FUNCTION OF THE NEMATODE *Caenorhabditis elegans*

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Abstract—The behavioral responses by the nematode *Caenorhabditis elegans* to 12 organic compounds was explored using tethered nematode and computer tracking methods. Results indicate that the nematode is attracted to acetone, diethyl ether, isoamyl acetate, isoamyl alcohol, 2,4-pentanedione, and *n*-propanol. No responses were detected to acetaldehyde, acetylcholine, ethanol, formaldehyde, i-propanol, and valerate. Isoamyl acetate and acetone were found to be the most potent attractants eliciting minimal responses at concentrations near 10^{-10} M. The geometry and charge distribution of a single hypothetical receptor that would interact with all the compounds that elicited a response and none of the compounds that failed to elicit a response is explored.

Key Words—Acetone, active site, chemoreceptor, chemosensitivity, isoamyl acetate, metabolite, nematode, threshold, *Caenorhabditis elegans*.

INTRODUCTION

The ability of organisms to detect chemicals has been explored in numerous studies. These studies have indicated remarkable chemosensitivity in some cases. The lowest threshold for chemosensation of a compound in the liquid phase was demonstrated by spermatozoa of the alga *Scytosyphon lomentaria*, which exhibited chemotaxis to conspecific sex pheromone concentrations as low as 6×10^{-13} M (Maier and Muller, 1986). The lowest threshold for chemosen-

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sation of compounds in the vapor phase may occur in lepidoperans. For example, the Indian meal moth, *interpunctella* (Mankin et al., 1980), exhibited chemotaxis to pheromone vapor phase concentrations as low as 2×10^4 molecules/cm^3. These thresholds are close to the limits of what is physically possible (Dusenbery, 1992a).

Further understanding of the mechanisms and functions of threshold chemosensation would be facilitated by studying an organism in which genetic chemosensory mutations can be isolated. These studies could probably be accomplished with the nematode *Caenorhabditis elegans*, which is now probably the most thoroughly characterized animal (Wood, et al., 1988). *C. elegans* is a soil nematode that is approximately 1 mm long, around 90 μm in diameter, and eats bacteria.

Although chemoreception has been studied in *C. elegans* for over 20 years (Dusenbery, 1973, 1974, Ward, 1973), none of the identified stimuli have been effective at concentrations sufficiently low to suggest a high specificity of binding to a receptor (Dusenbery, 1980b). The very high degree of potency (chemotaxis at 6×10^{-9} M) reported for chemotaxis of the nematode *Panagrellus redivivus* to amyl acetate (Balan, 1985) prompted us to look at the responses of *C. elegans* to related chemicals. More recently, a wide variety of polar organic molecules has been found to be effective in *C. elegans* chemotaxis (Bargmann et al., 1993), but threshold concentrations were not reported. Here we report on threshold determinations for several of these chemicals.

METHODS AND MATERIALS

Two different methods were utilized to determine the threshold for chemosensation by *C. elegans*. The first involved holding the nematode by its tail and pumping test solutions past it while recording its behavior with an oscillograph (Dusenbery, 1980a). These studies are referred to as "tethered nematode studies." In the second method, air containing a test compound was blown over the nematodes whose movements were tracked by a computer using a television camera for data input (Dusenbery, 1985, 1992b). These studies are referred to as "computer tracking studies." In order to verify behavioral analysis for isoamyl acetate, attraction studies were performed that involved comparing the number of nematodes that migrated towards the stimulant with the number that failed to do so on an agar surface.

All experiments were performed with young adult nematodes. The nematodes were obtained by transferring, three days prior to experimentation, approximately 500 N2 dauers (nematode larvae) to a bacterial lawn of *E. coli* (strain OP50) grown on NGM agar (Brenner, 1974).

A common problem in chemotaxis assays is false-positive results due to

contamination or ambient physical stimuli such as temperature (Pline et al., 1988). Consequently, protocols to guard against these problems were employed.

Tethered Nematode Studies. These experiments were used to test the *C. elegans* response to isoamyl acetate and acetylcholine. Concentrations tested and number of runs performed are presented in Table 1. Each experiment was initiated by placing one nematode on a testing platform on the observation stage of a microscope (Dusenbery, 1980a). The nematode was captured by its tail utilizing vacuum applied through a micropipet. Then it was moved into the testing chamber and situated so that its image fell on a series of photocells. The signal from the photocells was amplified and sent to an oscillograph to provide a graphical record of the nematode's movement.

Control runs were performed by filling the two syringe pumps with N-100 buffer [5.84 g NaCl, 0.50 g K_2HPO_4, and 3.00 g KH_2PO_4 added to 1.0 liter dH_2O (deionized water)] and alternately pumping the buffer from the two pumps past the nematode for 90-sec periods. One pump was designated the stimulus pump and the other as the control pump. One cycle was considered to consist of flow from the stimulus pump for 90 sec followed by flow from the control pump for 90 sec. Ten such cycles constituted a run. Two control runs were performed followed by two test runs. Test runs were performed in the same manner as control runs except that the stimulant pump contained N-100 buffer plus a known amount of test compound.

C. elegans exhibits several characteristic behavioral patterns that were utilized for determining chemosensation. The nematode usually moves forward via backward waves that are backwardly propagated undulations of the body (Croll, 1975). Forms of avoidance behavior include forward waves, body kinks, and coiling. Forward waves are referred to as a reversal bout if several come in succession.

Nematode behavior during a run was analyzed by counting the periods of avoidance behavior and noting which pump was actuated when each occurred, and whether a test run or a control run was being performed. Data for test runs were normalized by dividing the number of avoidance behavior periods when each pump was on during each test run by the number of periods of avoidance behavior displayed during the two control runs when the same pump was on. For each test run, a response value (R) was calculated such that $R = 1.0 - 2.0\ [A_s/(A_s + A_c)]$ where A_s is the normalized number of periods of avoidance behavior displayed when the stimulus pump was on, and A_c is the normalized number of periods of avoidance behavior displayed when the control pump was on. Possible values for R are between -1.0 and 1.0.

Computer Tracking Studies. These studies were used to confirm the nematode's response to isoamyl acetate and to detect possible responses to the rest of the compounds tested except acetylcholine (see Table 1). A suspension of nematodes was obtained by washing nematodes from the bacterial lawn on which

TABLE 1. RESPONSES AND STATISTICS[a]

Compounds tested	Res.	Results (number of positive runs/number of negative runs) 10^{-15} M	10^{-14} M	10^{-13} M	10^{-12} M	10^{-11} M	10^{-10} M	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M	*t* test α_{min}	Bin. Prob.
Acetaldehyde	No							1/1	3/4	3/4	3/4	2/0		
Acetone	Yes			1/3	1/3	3/1	**6/0**	4/2	6/0	2/0			0.025	0.016
Acetylcholine**	No								3/1	2/2	1/1	2/0		
Diethyl ether	Yes					1/1	2/0	4/0	**6/0**	6/0	6/0	2/0	0.010	0.016
Ethanol	No							1/1	0/2	1/1	2/2	2/0		
Formaldehyde	No						2/0	1/1	0/2	2/2	2/0			
Isoamyl acetate	Yes		3/5	5/3	4/6	6/4	**7/1**	6/0	4/0	2/0	1/1		0.050	0.031
Isoamyl acetate**	Yes				1/4	6/11	**12/1**	19/0	12/3	17/0	19/3	5/0	0.025	0.002
Isoamyl alcohol	Prob.							1/1	1/1	2/0	**4/0**	2/0	0.050	0.063
2,4-Pentanedione	Yes							2/2	3/4	4/0	**6/0**	2/0	0.005	0.016
i-Propanol	No								2/2	4/0	3/4	3/4		
n-Propanol	Yes								2/0	4/0	3/1	**6/0**	0.005	0.016
Valeric acid	No	0/2	2/2	2/2	2/2	3/1	2/2	0/4	0/4	1/1	1/1			

[a]Concentrations are given in moles per liter. The number of positive runs over the number of negative runs are listed for each concentration tested of a given compound. Responses considered to represent threshold are **in bold.** Statistical analysis applies to threshold responses. Compounds designated with double asterisks (**) represent tethered nematode studies while the rest of the compounds were studied via computer tracking studies.

they were grown. Bacteria were removed by allowing the nematodes to settle out of solution and siphoning off the supernatant. The nematodes were then washed with N-50 buffer (2.92 g NaCl, 0.50 g K_2HPO_4, and 3.00 g KH_2PO_4 added to 1.0 liter dH_2O), and the procedure was repeated until the concentration of bacteria was considered negligible. The nematodes were then transferred to glass plates on which tracking agar (0.09 g NaCl and 0.30 g Bacto Agar added to 30 ml dH_2O) had been poured in a thin layer covering roughly 50 cm^2. The nematodes were allowed to acclimate to their new environment for about half an hour before experiments were started.

Experiments were initiated by transferring the plates that contained the nematodes to the stage under the camera. The stage was designed in such a manner that when the glass plate was placed on it, a chamber was formed through which air could be passed (Pline and Dusenbery, 1987). The air that passed through the chamber and over the nematodes was first equilibrated with a solution in one of two sparging bottles. During control runs, both sparging bottles contained dH_2O. For test runs, one bottle contained dH_2O while the other contained dH_2O plus a known amount of test compound.

During a run, the computer kept track of the rate of movement of nematodes, with 50–100 nematodes usually being monitored. As in the tethered nematode studies, each run consisted of a series of cycles and in each cycle nematodes were exposed first to air from one bottle for 50 sec followed by air from the other bottle for 50 sec. Six cycles constituted one run, with the first cycle being treated as an acclimation cycle (no data collected).

The data set from each run was the rate of locomotion of the nematodes averaged over the five cycles. Average rates of locomotion were displayed in 5-sec intervals, yielding a representation of the average rate of locomotion as a function of time. Data from test runs were normalized by dividing rates of locomotion by the average rate of locomotion during corresponding time intervals in the two control runs. Next, the average normalized rate of locomotion during each half cycle was calculated by summing the ten 5-sec intervals and dividing by 10. The value (R) of the response of the nematodes to the stimulant was then calculated as $R = 2.0\ [L_s/(L_s + L_C)] - 1.0$ where L_s is the average normalized rate of locomotion during the stimulus half cycle, and L_c is the average normalized rate of locomotion during the control half cycle.

Attraction Studies. These experiments were performed with isoamyl acetate in order to verify that the behavioral patterns explored above represent nematode attraction to this compound. Attraction assays were performed as follows. First, plain agar was poured into 10-cm-diameter Petri plates. After the agar cooled, 2-cm-long sections of 50 μl micropipets were filled with dH_2O or with a nearly saturated solution of isoamyl acetate in dH_2O. The micropipet segments were placed at opposite sides of the Petri plate, and a drop of N-50 buffer containing around 100 nematodes was placed at the center of the Petri plate. The nematodes

were allowed to migrate for 15 min. Nematodes found within 0.5 cm of a given micropipet segment were counted.

Vapor Phase Concentrations. The mole fraction (y) of isoamyl acetate in the vapor phase that flowed over the nematodes during computer tracking studies was estimated for $y = x\gamma p_A/\phi P_s$ where x is the liquid phase mole fraction of isoamyl acetate; γ is 3830, the activity coefficient of isoamyl acetate that was calculated via UNIFAC (Fredenslund et al., 1975); p_A is 4 mm Hg, the partial pressure of isoamyl acetate at experimental temperature; ϕ is 1.0, the fugacity coefficient of isoamyl acetate; and p_s is the pressure of the system, taken to be one atmosphere. The number of molecules of isoamyl acetate per cubic centimeter (n) was estimated as $n = (yP_s/ZRT)\ (6.023 \times 10^{23})/1000$, where all variables are as above and Z is the compressibility factor, R is the gas constant, T is the experimental temperature, 6.023×10^{23} is Avogadro's number, and 1000 converts from molecules per liter to molecules per cubic centimeter.

Statistical Analysis. Student's one-tailed t test and binomial probability were utilized to verify significant results. The 95% confidence intervals and minimum values of α (α_{min}), which yielded 95% confidence levels, were calculated with Student's one-tailed t test. Values of $\alpha_{min} \leq 0.05$ were taken to indicate significant results. Binomial probability was based upon an assumed probability of getting a positive result being equal to 50% in the absence of an induced response. Binomial probabilities less than ≤ 0.05 were again taken to indicate significant results.

RESULTS

The results of experiments with isoamyl acetate are shown in Figure 1 (tethered nematode studies) and Figure 2 (computer tracking studies). Figure 1 indicates that the nematodes demonstrated a minimal response when the concentration of isoamyl acetate reached 10^{-10} M (theoretically 9×10^{-11} M). This low threshold was confirmed via computer tracking studies as shown in Figure 2. Figure 3 shows that the nematodes probably also demonstrated a minimal response to acetone at an acetone concentration of 10^{-10} M (theoretically 8×10^{-11} M). Table I indicates the statistical significance of these results.

Table I also shows that the results indicate chemosensation of diethyl ether, 2,4-pentanedione, n-propanol, and probably isoamyl alcohol. It should be noted that actual threshold for chemosensation of these compounds may be lower than indicated. Responses were not detected to acetaldehyde, acetylcholine, ethanol, formaldehyde, i-propanol, and valeric acid.

Attraction studies (eight repetitions) showed an average of 86 (± 6.0 SD) nematodes around the micropipet that contained isoamyl acetate for every 22

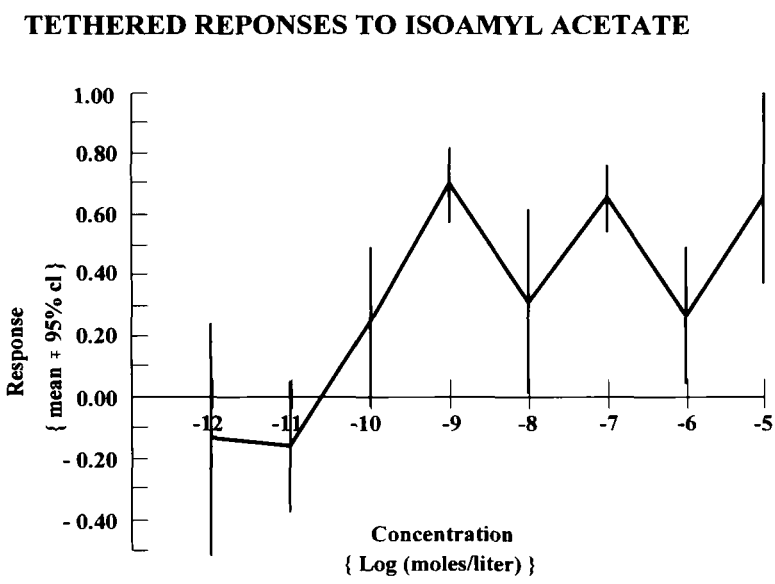

FIG. 1. The response demonstrated by *C. elegans* upon exposure to isoamyl acetate during tethered nematode studies. The data indicate that threshold response occurs at an isoamyl acetate concentration around 9×10^{-11} M. In this and the following figures vertical bars indicate the 95% confidence intervals according to Student's one-tailed *t* test.

(±9.5 SD) nematodes found around the micropipet that contained dH_2O only. The relatively high number of nematodes found around the latter micropipet was due to nematodes becoming entrapped in the meniscus formed by water at the interface between the micropipet and the agar surface. These results indicate that the behavioral response demonstrated by *C. elegans* to isoamyl acetate is an attractive response.

DISCUSSION

Caenorhabditis elegans demonstrated a remarkable response to both acetone and isoamyl acetate, which interestingly, has a pleasant aroma to humans and is known as pear or banana oil (Merck Index, 1968, p. 578). Isoamyl acetate was tested because Balan (1985) found amyl acetate to be a very potent stimulant of the nematode *P. redivivus*. The experiments performed with *C. elegans* indicated that isoamyl acetate elicited a response at a concentration of around 10^{-10} M (theoretically 9×10^{-11} M), while the experiments performed by

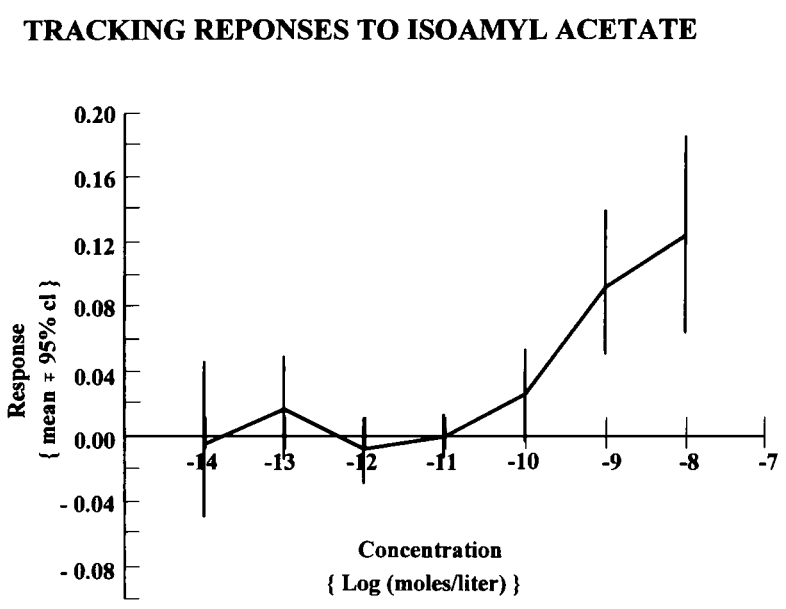

FIG. 2. The response demonstrated by the nematodes upon exposure to isoamyl acetate during computer tracking studies. The graph shows that data for computer tracking studies also indicated that the nematodes can respond to an isoamyl acetate concentration around 9×10^{-11} M.

Balan indicated that threshold response occurred at a concentration of 10^{-8} M (theoretically 6×10^{-9} M).

The difference in observed thresholds may depend more on the techniques used than on an inherent difference in the sensitivity of the two species. The experimental procedures employed in determining the threshold for *C. elegans* allowed free movement of the nematodes, while Balan's methods required that the nematodes accumulate in and under agar plugs containing the stimulant. The free movement of *C. elegans* in a liquid would allow for a higher rate of transfer of the stimulant to the sensilla than would occur when nematodes accumulated in agar plugs where the diffusivity of the solute would be lower and increased transfer due to convective currents would be absent. There is also a possibility of decreased availability of the solute due to absorption by the agar matrix, and it is possible that in Balan's experiments the nematodes were required to sense the stimulant at lower concentrations between the agar plugs.

Estimation of the vapor-phase concentration of isoamyl acetate at threshold indicated that it was around 10^9 molecules per cubic centimeter (theoretically

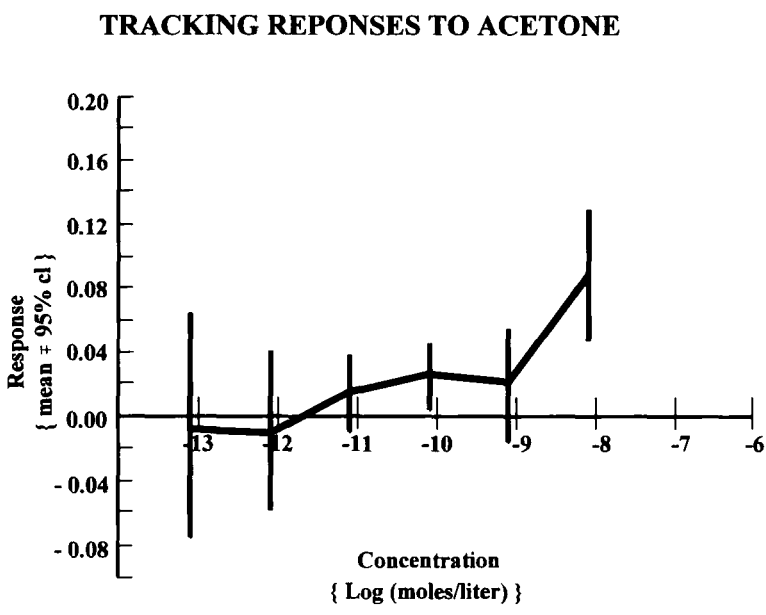

FIG. 3. The response profile demonstrated by *C. elegans* upon exposure to acetone during computer tracking studies. The graph shows that the nematodes may be sensing acetone concentrations as low as 8×10^{-11} M.

8×10^8 molecules/cm^3). This value is much higher than that found for the Indian meal moth *Plodia interpunctella*, which exhibited responses at a threshold concentration of 2×10^4 molecules/cm^3 (Mankin et al., 1980). This apparent difference in chemosensitivity probably results from differences in the size of the chemosensory organs (Dusenbery, 1992b).

It is interesting to consider why *C. elegans* would be so sensitive to isoamyl acetate when it is not a common bacterial metabolite. One possible explanation may be found in the fact that isoamyl acetate is commonly found in fruit (Gallander, 1974). It is plasuible that decaying fruit (the process of which would release isoamyl acetate into the local environment) represents a rich source of bacteria upon which the nematode feeds and also provides an important environment for the reproduction of *C. elegans* (Dusenbery, 1980b). This possibility is supported by two facts. First, *C. elegans* is a species specialized for the rapid exploitation of temporary resources due to its self-fertilization and rapid growth. Second, *C. elegans* is not found in general surveys of soil nematodes but appears in baits of decomposing vegetable matter (Dusenbery, 1980b).

Another interesting observation lies in the fact that short-chain acids, alco-

hols, and ketones (especially acetone) are common bacterial products under anaerobic conditions and that even though the macroscopic environment may be aerobic, the microscopic environment of a bacterial colony can be anaerobic, inducing the production of these products. The possibility that isoamyl acetate interacts with the same receptor that detects acetone was postulated when an experiment indicated that the nematodes responded to isoamyl acetate, isoamyl alcohol, and acetone, but not to valeric acid. These results indicate that, if these compounds were interacting with the same chemoreceptor, its active site would be composed of two hydrophobic regions on opposite sides of an electropositive crevice, which is 2–3 Å across. Actuation of the receptor would occur via simultaneous hydrophobic interactions between the two hydrophobic regions of the active site and hydrophobic regions of the stimulatory molecule. The electropositive crevice may act to help properly orient the stimulatory molecule. It may also assist in the actuation of the receptor if it consists of more than one properly oriented electropositive amino acid residues (i.e., electrorepulsion would become electroattraction in the presence of an electronegative oxygen atom). This hypothetical description and operation of the active site would explain the nematodes' attraction to appropriate polar organic molecules. This hypothesis was tested by predicting that diethyl ether, 2,4-pentanedione, and *n*-propanol would elicit a response from the nematodes and that the remainder of the compounds tested would not. Experimental results conformed with the prediction.

Several lines of experimental evidence indicate that the above mechanism of receptor actuation is correct and that all of the molecules that elicited a response from the nematodes are interacting with the same receptor. Support for the prediction that the active site operates via hydrophobic interactions can be gleaned from observing that the response to isoamyl acetate, which is a highly hydrophobic molecule, is stronger at any given concentration than is the response to acetone, which has low hydrophobicity (R = 0.091, 0.124, 0.181 for isoamyl acetate and R = 0.020, 0.087, 0.081 for acetone at 10^{-9} M, 10^{-8} M, 10^{-7} M, respectively). This result would be expected if the molecule operated via hydrophobic interactions. The postulate that both hydrophobic regions of the active site must simultaneously interact with hydrophobic regions of the stimulatory molecule is supported by observing that all of the molecules that elicited a response from the nematodes are capable of doing so, as shown in Figure 4, and that none of the compounds that failed to elicit a response from the nematodes would be able to do so, as shown in Figure 5. The possibility that all of the molecules that elicited a response from the nematodes are interacting with the same receptor is supported by comparing thresholds elicited in Table 1 with how easily a given molecule would be able to activate the receptor, as shown in Figure 4. If these molecules were interacting with separate receptors, each of which was adapted to detect a specific molecule, one would expect thresholds to be more or less uniform since interactions between each receptor and its

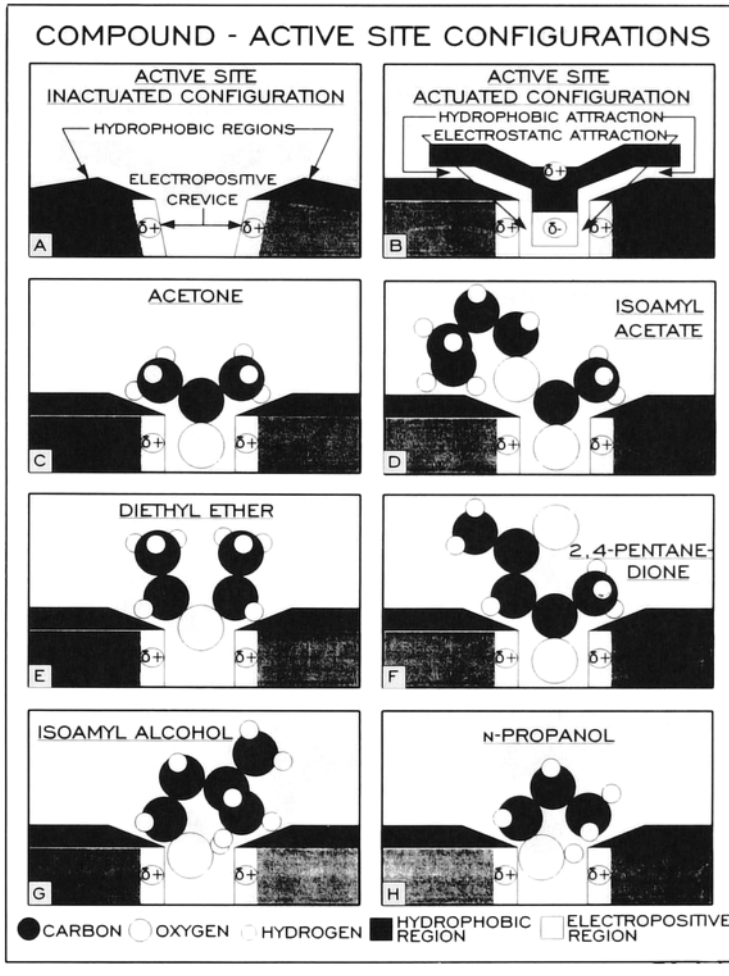

FIG. 4. A chemoreceptor whose active site was composed of two hydrophobic regions that were opposed to each other by 180° around an electropositive crevice 2–3 Å across would be able to interact with all of the molecules eliciting a response from the nematodes in an appropriate manner (i.e., simultaneous interaction between hydrophobic regions of the ligand and hydrophobic regions of the active site). These interactions would produce a conformational change in the receptor resulting in its activation.

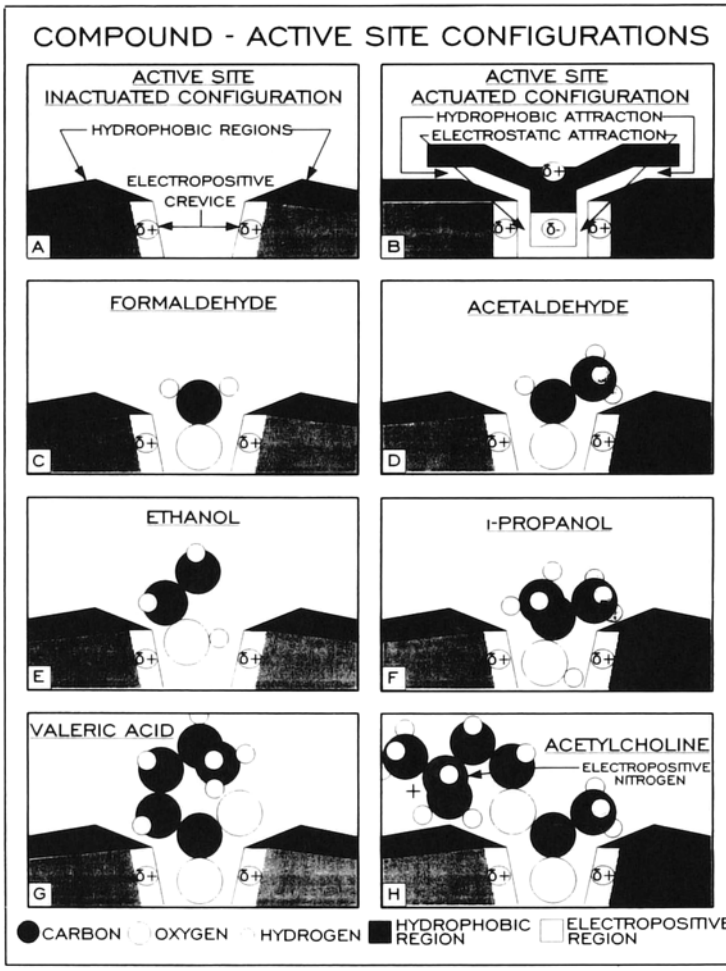

FIG. 5. All of the compounds that failed to elicit a response from the nematodes would be incapable of simultaneously interacting with both hydrophobic regions of the active site. Steric constraints and hydrophilic considerations prevent these interactions for i-propanol, valeric acid, and acetylcholine, respectively.

ligand should be highly proficient. However, what the above comparison indicates is that molecules that structurally have two hydrophobic groups opposed to each other around an oxygen atom, and therefore most easily interact with the active site, have the lowest thresholds. Molecules that must assume a restricted configuration in order to interact have higher thresholds, and the greater

the restriction on the configuration, the higher the threshold. This result conforms with the molecules interacting with the same active site. Further exploration of the nature of this chemoreceptor via additional testing, model building, and possibly amino acid sequencing would indeed be an interesting project.

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9-METHYLGERMACRENE-B; PROPOSED STRUCTURE FOR NOVEL HOMOSESQUITERPENE FROM THE SEX PHEROMONE GLANDS OF *Lutzomyia longipalpis* (DIPTERA: PSYCHODIDAE) FROM LAPINHA, BRAZIL

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Abstract—The principal volatile component (99+%) of the sex pheromone glands of *Lutzomyia longipalpis* from Lapinha, Brazil, has been isolated and characterized as a novel homosesquiterpene with the specific structure proposed as 9-methylgermacrene-B, (*E*,*E*)-8-(1-methylethylidenyl)-1,5,10-trimethyl-1,5-cyclodecadiene.

Key Words—Sex pheromone, tergal gland, *Lutzomyia longipalpis*, gas chromatography, mass spectrometry, high-performance liquid chromatography, nuclear magnetic resonance, homosesquiterpene, 9-methylgermacrene-B.

INTRODUCTION

The sandfly, *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae) is the vector of the protozoan parasite *Leishmania chagasi* (Cunha & Chagas) (Kinetoplastida: Trypanosomatidae), the causative agent of visceral leishmaniasis (VL) in the New World.

Male *L. longipalpis* release a sex pheromone from glands that appear as pale patches on the third or alternatively the third and fourth tergites of the

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abdomen (Lane et al., 1985). Studies on the morphology of these glands have shown that they are highly vacuolated from four days after emergence onwards (Boufana, 1990). Hexane extracts from the glands placed on filter paper disks attracted females (Ward et al., 1989) from distances of up to 60 cm (Morton and Ward, 1989). Hamilton et al. (1994) showed that in a population of sandflies collected in Jacobina, Brazil, the principal volatile component (90+%) of the tergal gland extract was responsible for sex pheromonal activity. The single component composition of the pheromone was supported by Dougherty et al. (1994), who showed that only one type of olfactory cell in the ascoid sensilla on the female antenna was stimulated by the sex pheromone gland extract.

L. longipalpis is known to exist as a species complex. Coupled gas chromatography–mass spectrometry (GC-MS) of pheromone gland extracts (Lane et al., 1985; Phillips et al., 1986) showed that there were two populations of *L. longipalpis* producing single compounds. One population produced a compound with a molecular weight of 218, whereas the other population produced a compound with a molecular weight of 272. These molecular weights and the fragmentation patterns suggested the components were homosesquiterpene ($C_{16}H_{26}$) and diterpene ($C_{20}H_{32}$) hydrocarbons. The former showed similarity to the homofarnesene isolated from the Dufour's gland of *Myrmica rubra* (Attygalle and Morgan, 1982).

GC analysis of pheromone gland extracts prepared from *L. longipalpis* from a wide geographic range in South America indicated that there could be up to six different populations of this sandfly (Hamilton and Ward, 1991). These populations fall within the two chemically distinct classes suggested by Lane et al. (1985) and Phillips et al. (1986) and represent sibling species.

The objective of this current work was to characterize structurally the major compound in the pheromone of *L. longipalpis* from Lapinha, Brazil.

METHODS AND MATERIALS

Sandflies. Sandflies were obtained from Gruta da Lapinha, Minas Gerais, Brazil (19.34S, 43.58W). They were colonized according to the method of Modi and Tesh (1983) at a temperature of 26°C, 98% relative humidity, and a 12:12LD photoperiod. Pheromone extract was prepared as described previously (Hamilton et al., 1994) from 4 to 5 day-old adult males. Samples were stored at −70°C in sealed glass ampoules prior to analysis.

For NMR analysis approximately 8000 males were collected in light traps suspended from the roof of a cave during eight nights at Gruta da Lapinha. Samples extracted in pesticide-grade hexane were stored at −70°C until transport to the UK in a 3DS dry shipper liquid nitrogen container. Extract was

filtered through glass wool, and solvent was reduced under N_2 to 1 male equivalent (ME)/μl prior to further analysis.

Column Chromatography. Column chromatography was performed on a silica gel microcolumn prepared by placing BioSil-A (20–44 μm) (BioRad, Hemel Hempstead, England) silica gel (0.25 g) in a Pasteur pipette. The column was precleaned by eluting sequentially with hexane (10 ml) (pesticide grade, BDH, Poole, England) followed by diethyl ether (10 ml) (chromatography grade, BDH). The sample was eluted in three column volumes of hexane.

High-Pressure Liquid Chromatography (HPLC). HPLC analysis was performed with a Waters HPLC system: 600E multisolvent pump and gradient controller, U6K injector, and 991 photodiode array detector (Waters, Watford, England). Analyses were carried out on two C18 analytical columns (3.9 × 300 mm) (Waters Resolve) in series with a C18 precolumn (Waters Resolve), heated to 30°C. The mobile phase was acetonitrile–isopropanol 70:30 at 1 ml/min. Standards used for calibration and monitoring of separations were (−)-β-caryophyllene and caryophyllene oxide (Aldrich, Gillingham, England) (1 μg/μl). Fractions for further GC, GC-MS, and NMR analysis were collected manually in 10 ml acid cleaned beakers. The nonpolar gland extract was recovered from the relatively polar mobile phase by a liquid–liquid partition (hexane–H_2O). For NMR studies deuterated cyclohexane (C_6D_{12}) was used as the organic phase in the liquid:liquid separation.

Gas Chromatography (GC). GC analyses were carried out using a J&W DB-1 capillary column (Alltech, Carnforth, England) (30 m × 0.32 mm ID, 0.25 μm phase thickness) in a Shimadzu model 15-A gas chromatograph (Shimadzu Corp, Kyoto, Japan) linked to a Shimadzu CR-5A data-pac integrator. Injection of extract was via a Grob split/splitless injector in the splitless mode (0.6 min sampling time) at 180°C. Carrier gas was helium at 1 ml/min. The GC was temperature programmed with an initial 2 min at 45°C, then a rise of 15°C/min to a final isothermal period at 310°C. Detector (FID) temperature was set at 310°C.

Preparative Gas Chromatography. Preparative GC was carried out using a HP1 megabore capillary column (Hewlett Packard, Stockport, England) (10 m × 0.53 mm ID, 2.65 μm phase thickness) in a Pye Series 105 gas chromatograph (Pye Ltd., Cambridge, England). Carrier gas was helium at 1 ml/min. The GC was temperature programmed with an initial 2 min at 45°C, then a rise of 15°C min to a final isothermal period at 310°C. Injector temperature was set at 180°C, and detector (FID) temperature was set at 310°C. For the collection of injected material, a column split of 99:1 was used. The majority of the effluent passed through a heated outlet and was collected in a precleaned (heated) capillary tube (100 mm × 2.0 mm). Collected material was eluted from the capillary tube for NMR analysis in deuterated chloroform.

Gas Chromatography–Mass Spectrometry (GC-MS). Coupled GC-MS was performed on a Hewlett-Packard 5880A GC with an HP-1 capillary column (50 m × 0.32 mm ID, 0.25 μm film thickness) directly coupled to a VG 70-250 MS (electron impact, 70 eV, 250°C). Sample was introduced via a cold (30°C) on-column injector. The GC was temperature programmed with an initial 5 min at 30°C, then a rise of 5°C min to a final isothermal period at 250°C. GC-MS studies were performed on crude extract from dissected glands, hexane fractions from a BioSil-A column, as well as on reduction, epoxidation, and rearrangement products.

Gas Chromatography–Infrared–Mass Spectrometry (GC-IR-MS). Further analysis was carried out using a Hewlett Packard 5890 II+ Gas Chromatograph coupled to a Hewlett Packard 5972A benchtop mass spectrometer (electron impact, 70 eV, 165°C). A Hewlett Packard 5965B Fourier transform infrared detector was placed in series between the GC and MS. Injection of the sample was via a splitless injector (110°C) (sampling time 0.7 min). Chromatographic analysis was on a HP-5 capillary column (3 m × 0.32 mm ID, 0.52 μm film thickness), IRD was connected to the MS via a capillary transfer line (1.2 m × 0.10 mm ID, 0.17 μm film thickness). Carrier gas was helium at 2 ml/min. Injector temperature was set at 120°C. The GC was temperature programmed with an initial 2 min at 45°C, then a rise of 20°C/min to a final isothermal period at 150°C. IR data were obtained with flow cell and transfer line temperatures set at 130°C.

IR and MS spectra for standards were obtained with a DB Wax capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness). Carrier gas was helium set at 1.5 ml/min with the injector set at 120°C. The GC was temperature programmed with an initial 50°C, then a rise of 10°C/min to a final isothermal period at 250°C. IR data were obtained with flow cell and transfer line temperatures set at 150°C.

Nuclear Magnetic Resonance (NMR). NMR spectra were recorded in deuteriochloroform ($CDCl_3$) or deuteriocyclohexane (C_6D_{12}) solution using tetramethylsilane as the internal standard. Proton NMR spectra (^1H NMR) were recorded at 400 MHz on a Jeol JNM-GX400 with Fourier transform facility. Carbon-^{13}NMR spectra (^{13}C NMR) were similarly recorded at 100 MHz.

Hydrogeneration. Ten male equivalents of *L. longipalpis* extract (10 μl) were placed in a small reaction vessel prepared from a Pasteur pipet. Palladium black (20 μg) (Aldrich Chem. Co.) catalytic reducing agent was added and the mixture was shaken. Hydrogen was bubbled through the solution for 2 min.

Expoxidation. Dimethyldioxirane (DMD) reagent was prepared as described by Adam et al. (1987). *L. longipalpis* extract (100 ME, 100 μl) was eluted from a Florisil (350 mg) pipette column in hexane (2 ml). The volume was reduced

under N_2 to 100 μl and the fraction then placed in a 1-ml vial and reagent (40 μl) added. The reaction was allowed to proceed for 24 hr before analysis by GC and GC-MS.

Rearrangement with Silicic Acid. Using a technique described by Nishino et al. (1977), 100 ME of HPLC purified extract (100 μl) was placed in a glass vial with silicic acid (100 μg). The preparation was sealed and stored at 37°C for 12 hr before GC-MS analysis.

Thermal Rearrangement. HPLC extract (100 ME, 100 μl) was heated to 180°C under N_2 and the products analyzed by GC-MS and NMR.

Standards. Germacrene-B was synthesized according to the method of Brown et al. (1967) and was also obtained from the hydrocarbon fraction of the peel of Key limes (*Citrus aurantifolia* Swingle) (Clark et al., 1987). Germacrene-D was similarly obtained from tansy (*Tanacetum vulgare* L.). Germacrone was obtained from rocksfoot cranesbill (*Germanium macrorhyzum* L.) (Ognjanoff et al., 1957; Herout et al., 1959).

Synthesis of Hexahydro-9-methylgermacrene (9-Methylgermacrane). Germacrone was treated with lithium diisopropylamide as base in dimethoxyethane and iodomethane in 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU) to give (±)-9-methylgermacrone, *m/z* 232(M^+, 12), 135(100), ^{1}H-NMR ($CDCl_3$) 1.21(d, 3H, J = 7) 1.41(s, 3H), 1.47(s, 3H), 1.69(s, 3H), 1.77(s, 3H), 2.0–2.5(complex, 4H), 2.87(m, 2H), 3.57(q, 1H, J = 7), 4.63(m, 1H), 5.05(m, 1H), ^{13}C NMR 11.1, 13.3, 15.7, 19.8, 22.3, 23.6, 29.7, 37.7, 57.7, 125.7, 130.0, 130.5, 132.2, 134.7, 138.4, 210.4. Purification was achieved on a Florisil column in a solvent gradient from hexane (100%) to hexane–diethyl ether (80:20) and crystallization from chilled methanol (mp 51°C). Hydrogenation was effected by Adams catalyst in acetic acid under hydrogen at room temperature and pressure to give hexahydro-9-methylgermacrone (as a mixture of stereo-isomers), *m/z* 238(M^+, 12), 55(100). A loss of olefinic proton and carbon absorbencies indicated complete hydrogenation. Conversion of the ketone to the corresponding methylene was achieved via reduction to the hexahydro-9-methylgermacrol (new absorbencies at 3.75 ppm in ^{1}H NMR and in the 75–78 ppm region in ^{13}C NMR, consistent with alcohol function) with lithium aluminum hydride in diethyl ether. Treatment with methanesulfonyl chloride gave elimination products, showing olefin absorbencies at 4.75–4.85 ppm in ^{1}H NMR and 129–140 ppm in ^{13}C NMR, comprising a mixture of the tetrahydro-9-methylgermacrenes. These olefins were hydrogenated with 5% palladium on charcoal in ethanol under a hydrogen atmosphere at ambient temperature and pressure to give hexahydro-9-methylgermacrene (9-methylgermacrane). On GC-MS a cluster of four peaks was observed that gave very similar spectra. Loss of olefinic absorbencies in the IR spectra indicated complete hydrogenation.

RESULTS

GC. GC analysis of extract prepared from dissected pheromone glands gave the typical chromatogram seen in Figure 1A. Two dominant peaks at 11.6 (*a*) (37%) and 12.3 (*b*) (63%) min were seen. By comparison the retention times (R_t) of pentadecane and hexadecane were 11.5 and 12.2 min, respectively. Peak *b* had a conspicuous leading edge, while the presence of other small peaks between 10 and 16 min indicated the presence of other material in trace amounts.

HPLC. HPLC analysis of pheromone gland extract produced the typical chromatogram seen in Figure 1B. The chromatogram was dominated by a single peak (*c*) at 10.7 min (λ_{max} = 203.4 nm) (99%). Caryophyllene (λ_{max} = 208.2 nm) and caryophyllene oxide (λ_{max} = 209.5 nm) had R_t of 7.16 and 10.3 min, respectively. The large peak (10.4–11.2 min) was collected as was eluent from before (8–10.4 min) and after (11.2–13.0 min) the main peak. GC analysis of these fractions indicated that the large HPLC peak (*c*) gave rise to the main GC peaks *a* and *b* at 11.6 and 12.3 min in the same approximate proportion as the crude extract (33–66%). Tests on the caryophyllene standard indicated that preparative HPLC and subsequent back extraction into hexane was between 60 and 90% efficient.

GC-MS. The recovered large HPLC peak (*c*) was injected via cold on-column injector, and one homogeneous but symmetrically diffuse peak was obtained. The mass spectrum of this material is given in Figure 2. On reduction this material gave a cluster of several isomers separating into four peaks with similar spectra. The major peak was compound *d*, *m/z* 224(M^+, 8), 181(7), 125(32), 111(53), 97(85), 83(86), 69(100), 57(97), 55(100), 43(65). Epoxidation of the natural material gave compound *e*, *m/z* 266(M^+, 1), 248(2), 123(28), 109(33), 95(45), 85(32), 84(31), 83(32), 69(55), 55(65), 43(100). Two silicic acid rearrangement products were seen, compound *f*, *m/z* 218(M^+, 99), 175(72), 203(66), 105(62), 95(62), 119(60), 95(60), 133(55), 81(39), 67(35), 55(52), 43(28), and compound *g*, *m/z* 218(M^+, 74), 175(100), 133(43), 122(52), 107(70), 95(85), 81(52), 55(58), 67(38). The heat-treated parent compound (*c*) gave three products eluting as sharp peaks. The first of these (*h*), unlike the parent compound, gave a sharp peak that eluted (R_t = 38.7 min) before the parent compound (R_t = 40.3 min), *m/z* 218(M^+, 10), 203(15), 175(16), 161(10), 147(13), 135(50), 121(100), 107(60), 93(76), 79(31), 67(50), 55(33), 41(62). Two closely eluting compounds (*j* and *k*) were seen at R_t = 41.7 min, *m/z* 218(M^+, 85), 203(31), 189(8), 175(78), 161(19), 133(68), 119(67), 105(65), 95(72), 55(60), 41(100), and at R_t = 42.0 min, *m/z* 218(M^+, 60), 203(36), 189(14), 175(93), 149(37), 122(55), 107(88), 95(89), 81(54), 67(46), 55(55), 41(100).

GC-IR-MS. Infrared spectra for the putative pheromone from *L. longipalpis*

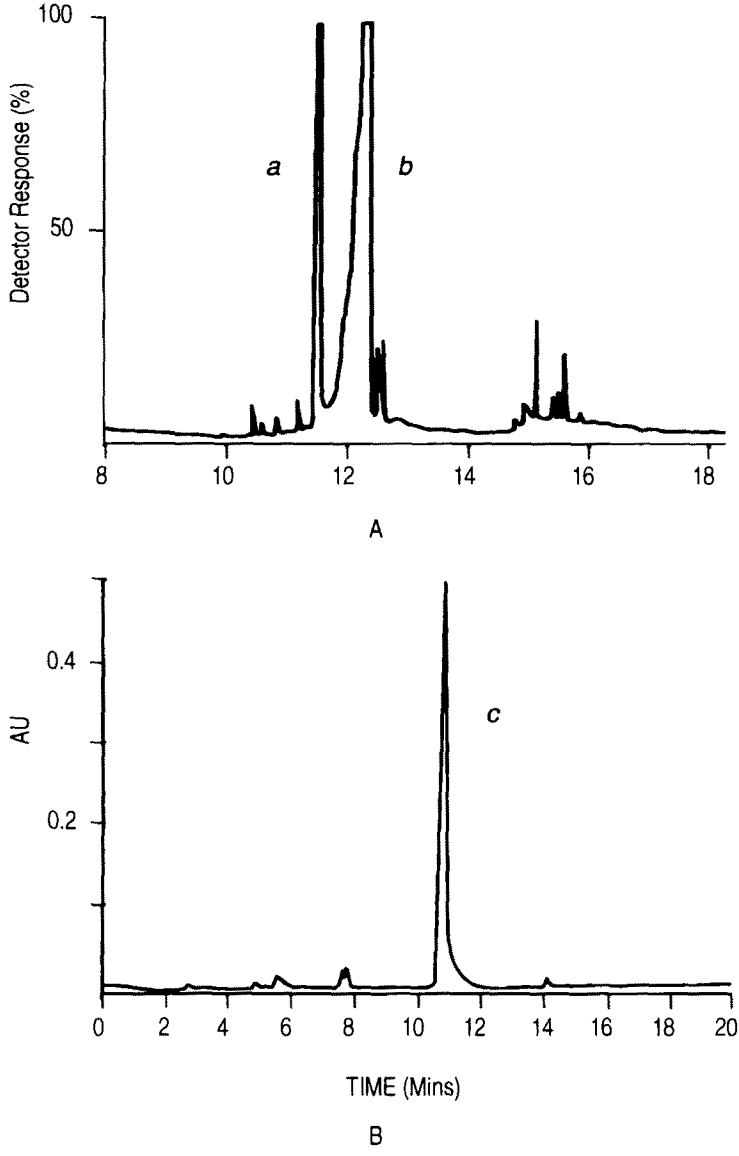


FIG. 1. GC (A) and HPLC (B) trace of hexane extract of dissected sex pheromone gland of male *L. longipalpis*.

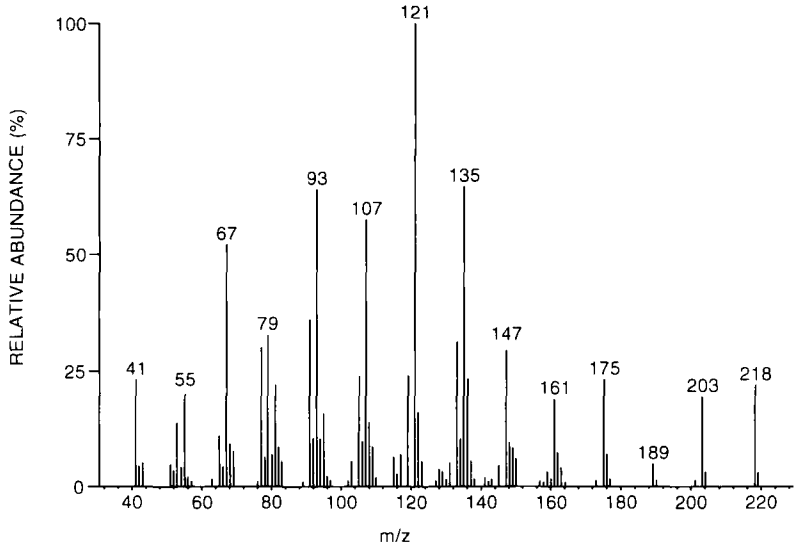


FIG. 2. Mass spectrum (cold on-column GC-MS) of main component *c*, of extract of sex pheromone gland of male *L. longipalpis*.

Lapinha and the reduced product are given in Figure 3A and B. Peaks at 2362 and 2336 cm^{-1} are due to CO_2.

NMR. For untreated putative pheromone from *L. longipalpis* (*c*): ^1H NMR (C_6D_{12}) 0.94(d, 3H, J = 7), 2.98(d, 1H, J = 14), 4.30(d, 1H, J = 11), 4.62(d, 1H, J = 11). Solvent impurities obscured the highfield region between 1.2 and 1.6 ppm. ^1H NMR ($CDCl_3$) 0.96(d, 3H, J = 7), 1.36(s, 3H), 1.46(s, 3H), 1.61(s, 3H), 1.63(s, 3H), 1.8–2.3(complex, 8H), 2.98(d, 1H, J = 14), 4.30(d, 1H, J = 11), 4.62(d, 1H, J = 11).

For preparative GC thermolysis products from pheromone gland extract: ^1H NMR ($CDCl_3$). The major component showed singlet absorbencies at 4.52 and 4.77 ppm and a singlet absorbency at 5.36 ppm. The minor component had absorbencies at 4.60(s, 1H), 4.85(s, 1H), 4.86(d, 1H, J = 16), 4.98(d, 1H, J = 10), 5.56(dd, 1H, J = 16, 10).

For thermal rearrangement of 9-methylgermacrone: ^1H NMR ($CDCl_3$). The olefin region showed absorbencies at 4.72(s, 1H), 4.87(d, 1H, J = 17), 4.91(s, 1H), 5.04(d, 1H, J = 11), 5.66(dd, 1H, J = 17, 11).

DISCUSSION

HPLC analysis and elution from a Florisil column by hexane suggested a hydrocarbon structure for the putative sex pheromone. The single peak (*c*)

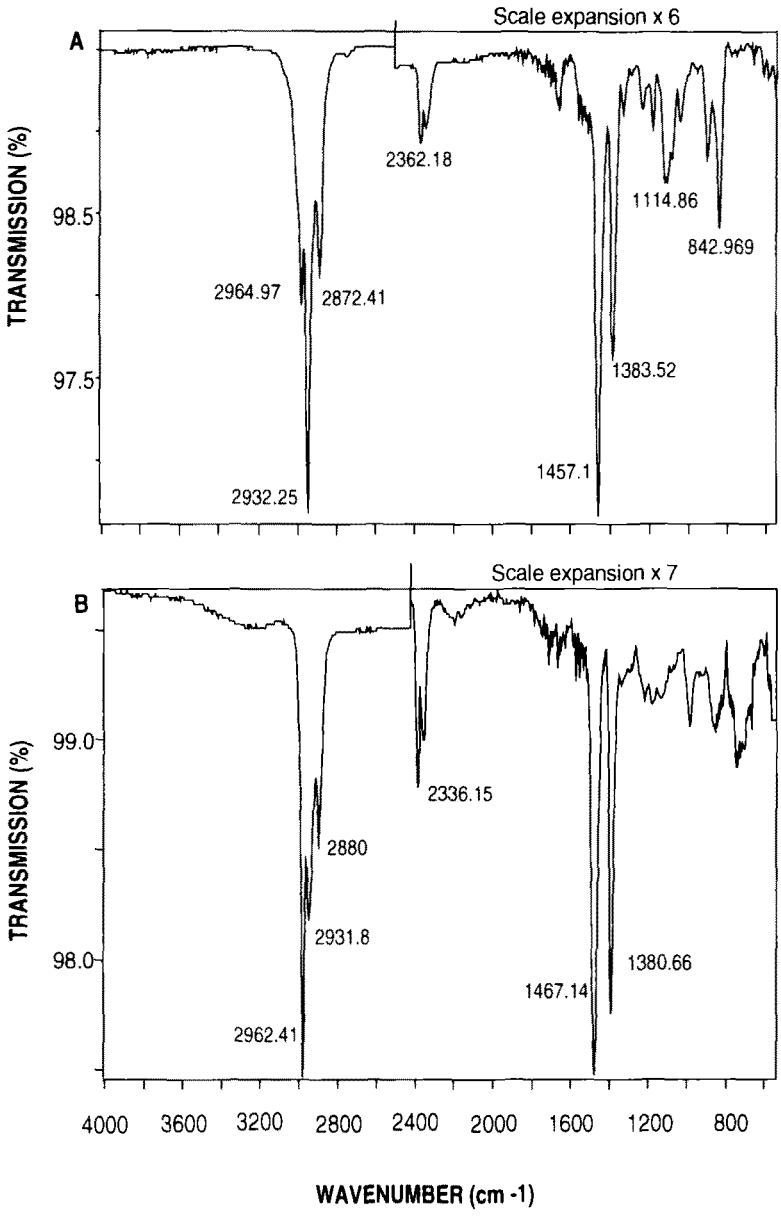
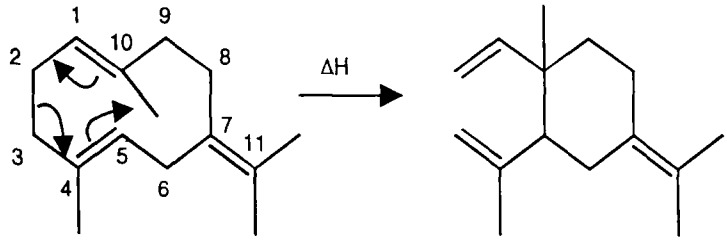


FIG. 3. Infrared spectra of (A) the main component *c* and (B) reduced main component *d* of extract of sex pheromone gland of male *L. longipalpis*.

obtained by HPLC was at variance with the two major components (*a* and *b*) suggested by GC. However, use of the cold on-column injector (30°C) gave only one peak that was symmetrically diffuse, also corresponding to *c*. These observations suggested thermal rearrangement taking place in the heated injector and during chromatography. The EI mass spectrum suggested a molecular weight of 218, which was confirmed by CI-MS. Reduction and epoxidation gave products (*d* and *e*) with molecular weights higher by 6 amu (224, M^+) and 48 amu (266, M^+), respectively, confirming the presence of three double bonds.

The EI mass spectrum for the putative sex pheromone has the ions *m/z* 175, 203, and 218 (M^+), each 14 amu higher than for ions of corresponding intensity in the spectrum of germacrene-B (I), a plant component previously known as germacratriene (Allen and Rogers, 1967; Hartley and Fawcett, 1969), i.e., 161, 189, 204 (M^+) (NIST, 1990), and showing strong similarities at lower *m/z* values. In addition the IR spectrum of *c* had similar features to that of germacrene-B (Clark et al., 1987). The mass spectrum of the putative sex pheromone was substantially different from those for the other germacrenes, A and D. This suggested a structure for this compound related to germacrene-B but with an extra methylene placed so as not to disrupt the main features of fragmentation during mass spectrometry.

Thermolytic rearrangement of a compound similar to that of germacrene-B (I), would explain why several peaks were obtained when the gland extract was analyzed on a GC with a heated injector. By comparison, when a cold on-column injector was used, a single peak was obtained that appeared diffuse through decomposition on the column during development of the chromatogram. Thermal rearrangement on heating at 180°C gave three main products with the earliest eluting peak (*h*) having a mass spectrum closely similar to that of the parent compound *c* but suggesting a structure similar to that for the elemene, elixene (II), which could arise from the thermal rearrangement of germacrene-B

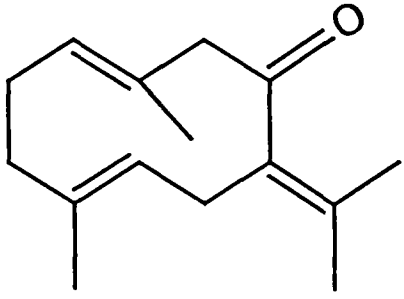


(I) (II)

(I) via a sigmatropic Cope rearrangement (Jones and Sutherland, 1968; Yoshihara et al., 1969; Weinheimer et al., 1970). The compounds eluting later, *j* and

k, appeared to be products of further cyclization of the germacrene-B ring structure. Germacrenes are also acid labile, and treatment with silicic acid gave two main products *f* and *g*, which had spectra with intense molecular ions suggesting bicyclic products that are known to result from rearrangement of germacrenes catalyzed by protonation (Hartley and Fawcett, 1969; Weinheimer et al., 1970).

The ^{1}H NMR (in $CDCl_3$) spectrum for *c* was similar to that for germacrene-B (Clark et al., 1987, Allen and Rogers, 1967). There also were similarities with the spectrum of germacrone (III). The most striking feature of the



(III)

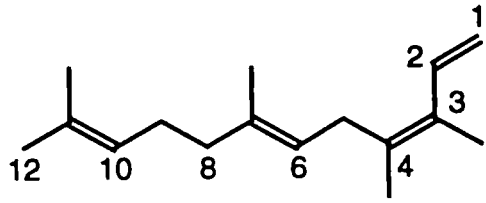
proton NMR spectrum of the putative sex pheromone (*c*) was the high field position of the olefinic protons at 4.62 and 4.30 ppm. These absorbances, as with germacrene-B (Clark et al., 1987), are very broadened doublets. The broadening is due to the transannular effects that hinder normal relaxation of the molecule in the NMR experiment and the appearance as only doublets arises through the *J* value being almost zero for one of the adjacent methylene protons.

^{1}H NMR (in $CDCl_3$) of the compounds obtained by preparative GC of the sex pheromone gland extract revealed the presence of two components arising from *c* by thermal rearrangement during GC. The major component (R_t = 12.3 min) had singlet olefinic absorbencies at 4.52, 4.77, and 5.36 ppm. The minor component (R_t = 11.6 min) was significant in that it had absorbencies at 4.60 and 4.85 ppm and absorbencies characteristic of an isolated vinyl group at 4.86(d, 1H, J = 16), 4.98(d, 1H, J = 10) and 5.56(dd, 1H, J = 16, 10). These NMR data supported a structure similar to elemene (II).

The position of the additional methylene on the sesquiterpene skeleton was suggested by a process of elimination and by taking into account biosynthetic considerations. The four allylic methyl groups analogous, and with similar NMR shift values, to those present in germacrene-B and germacrone, were at 1.36, 1.46, 1.61, and 1.63 ppm as singlets for *c*, demonstrating that the extra methylene was not substituted into a methyl of the germacrene-B structure. Indeed, the methylene comprised an extra methyl at 0.96 ppm and was as a doublet, *J*

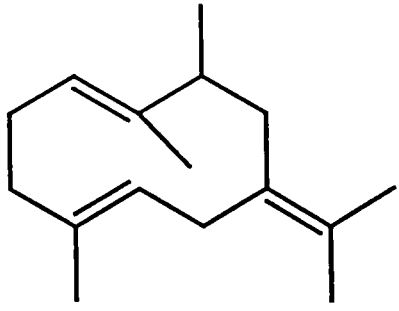
= 7, suggesting substitution at a ring methylene of the germacrene-B structure. Such substitution at carbon 6 could be eliminated as the absorbances at δ = 2.25 and 2.98 pm are correlated in the H-H COSY experiment. The possibility of substitution at carbon positions 2 or 3 was discounted because the elemene, corresponding to elixane (II) from the heat-treated putative sex pheromone, displayed five olefinic proton absorbances, whereas any substitution here would reduce the olefinic absorbencies to four.

Homosesquiterpenes, where encountered so far, fall into three biosynthetic classes, those arising from: (1) oxidative clevage products of diterpenoids (Gäbler et al., 1991); (2) juvenoids, i.e., with methylene substitution into the methyl positions; and (3) homofarnesenes with the methylene substitution at carbon 4, e.g., (*Z,E*)-α-homofarnesene (IV) (Ritter et al., 1977; Vander Meer, 1983,



(IV)

1986; Attygalle and Morgan, 1982, Jackson and Morgan, 1993). The first two are eliminated by spectroscopic properties already discussed. Biosynthetic class (3) would suggest substitution at carbon 3 in germacrene-B but has been discounted. However, position 9 in germacrene-B, i.e., position 8 in the homofarnesene (IV), would be biosynthetically analogous. This position is therefore favored over carbon position 8 for the pheromone component. The putative sex pheromone *c* structure is therefore proposed as 9-methylgermacrene-B, systematically named (*E,E*)-8-(1-methyl ethylidenyl)-1,5,10-trimethyl-1,5-cyclo decadiene (V).



(V)

Although germacrene-A comprises the alarm pheromone for the spotted alfalfa aphid, *Therioaphis maculata* (Nishino et al., 1977), and germacrene-D is a plant-derived sex stimulant for male American cockroach, *Periplaneta americana* (Tahara et al., 1975), neither germacrene-B or any homogermacrenes have been reported from insects.

Approaches were made to the synthesis of 9-methylgermacrene-B from 9-methylgermacrone, numbered here as for structure I, by the approach successfully used for germacrene-B by Brown et al. (1967). However, in the key step of the reaction pathway, lithium–ammonia reduction of 8-acetoxy-9-methylgermacrol yielded only the corresponding alcohol (unpublished). Nonetheless a degree of confirmation by synthesis was achieved when 9-methylgermacrone was reduced and deoxygenated to give a product that had the same MS and very similar IR to the hydrogenation product from the pheromone, i.e., compound *c*, and that gave peak enhancement on GC by coinjection.

There are four asymmetric carbons in hexahydro-9-methylgermacrene, i.e., 9-methylgermacrane, creating eight potentially separable diastereoisomeric pairs of enantiomers, but only four main peaks were resolved on GC. All of the natural product peaks were enhanced by coinjection of synthetic product and the spectra for the four peaks were similar within and between the two samples. Hexahydro-9-methylgermacrene gave a similar pattern of fragmentation to germacrane (NIST, 1990), with the higher mass ions differing by 14 amu.

Attempts are being made to confirm this proposed structure by synthesis and the absolute stereochemistry at carbon 10 will be determined. The characterization of the pheromones for two other populations of *L. longipalpis* is in progress.

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EFFECT OF SIMPLE PHENOLIC COMPOUNDS OF HEATHER (*Calluna vulgaris*) ON RUMEN MICROBIAL ACTIVITY IN VITRO

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Abstract—Heather (*Calluna vulgaris*), a common dietary component of many mammalian herbivores, contains a broad range of tannin phenolics, flavonoids, and simple phenolic compounds. These may lead to a number of negative effects on herbivores as a result of toxicity or digestive inhibition. We assessed the effect of some simple phenolic compounds from heather on rumen microbial activity using the *in vitro* consecutive batch culture technique. The compounds tested were orcinol, quinol, and arbutin, a glucoside of quinol. At a concentration of 10 mM, both orcinol and quinol depressed production of gas and volatile fatty acids (VFAs). Orcinol and, to a lesser extent, quinol also reduced digestion of the substrate. In contrast to these negative effects on rumen microbial fermentation, arbutin stimulated both gas and VFA production, probably due to the presence of the fermentable sugar moiety. The presence of arbutin also resulted in different concentrations and proportions of VFAs compared to the presence of orcinol and quinol. Since many simple phenolic compounds are found in plants in the form of glycosides, the negative digestive effects of the phenolic moieties may be accompanied by the positive effect of the sugar moiety, which is liberated in the gastrointestinal tract on hydrolysis by glycosidase enzymes. This study suggests that when phenolics are present as glycosides, they do not necessarily adversely affect digestion by herbivores.

Key Words—Heather, *Calluna vulgaris*, sheep, secondary compounds, phenolic, rumen, microorganisms, activity.

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INTRODUCTION

Plant secondary compounds are involved in complex interactions between plants and their biotic environment (Bell, 1981) and may be produced by plants to deter feeding by herbivores (Freeland and Janzen, 1974; Rhoades and Cates, 1976; Rosenthal and Janzen, 1979). One of the largest and most widespread groups of plant secondary compounds are the phenolics (Harborne, 1991), which sometimes are consumed in large quantities by herbivores, particularly those that utilize lignified or woody plants as part of their diet (McArthur et al., 1991). Their effects may be beneficial, although in large quantities they are considered to have a negative impact because they are either toxic or reduce digestibility (Scheline, 1991; Kumar and Singh, 1984), or both (Foley and McArthur, 1994). Many mammalian herbivores, including ruminants, utilize microbial fermentation to digest food substrates, producing volatile fatty acids (VFAs) from carbohydrates (Van Soest, 1983). In this study we investigate both positive and negative effects of some specific non-tannin phenolics using a batch culture technique that simulates a rumen-like fermentation in the laboratory.

Phenolic compounds may have a negative effect on digestion in a number of ways. Firstly, certain phenolic compounds, particularly tannins, have the ability to interact with proteins and carbohydrates, thus reducing the digestibility of these components of the diet (Hagerman et al., 1992; Barry and Duncan, 1984). They may also interact with digestive enzymes and reduce their activity (Makkar et al., 1988). Phenolic compounds can also affect digestion through their toxic effect on rumen miroorganisms (Akin, 1982). A number of previous *in vitro* studies have shown that phenolic acids that are associated with the carbohydrates of cell walls have an inhibitory effect on rumen microorganisms. The effects observed include (1) reduced bacterial growth rate (Akin, 1982; Borneman et al., 1986; Chesson et al., 1982; Hartley and Akin, 1989), (2) reduced colonization of plant material (Akin and Rigsby, 1985; Varel and Jung, 1986), leading to (3) reduction of digestibility of cellulose and hemicellulose (Akin, 1982; Akin and Rigsby, 1985; Borneman et al., 1986; Varel and Jung, 1986).

Heather (*Calluna vulgaris*) is a woody perennial plant found in many areas of the northern hemisphere and is a particularly dominant species in the European oceanic heathlands (Bunce and Barr, 1988). Many herbivores such as mountain hares (*Lepus timidus*) (Iason and Waterman, 1988), red grouse (*Lagopus lagopus scoticus*) (Savory, 1978), red deer (*Cervus elaphus*) (Gordon, 1989), and sheep (Milner and Gwynne, 1974) depend on heather as an important part of their diet, particularly during the winter when other food sources are scarce. Heather contains a large proportion of phenolic secondary compounds (Iason et al., 1993), including many non-tannin phenolics that may be present as simple phenolics or as phenolic glycosides (Harborne and Williams, 1969; Jalal et al.,

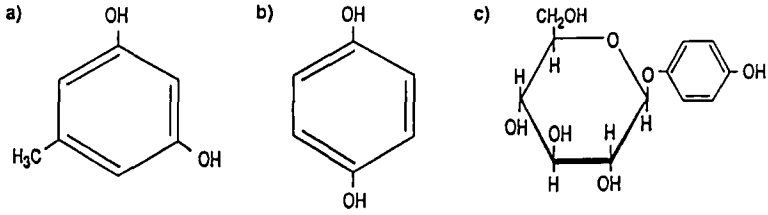


FIG. 1. The structure of: (a) orcinol, (b) quinol, and (c) arbutin.

1982). The aim of this study was to measure the effect of some of these compounds on rumen microbial digestion *in vitro*. The compounds tested were orcinol, quinol, and arbutin, the D-glucoside of quinol (Figure 1). Orcinol was chosen as it is present in *Calluna* shoots (Martin et al., 1983). Quinol is also present in *Calluna* shoots, in the form of its glucoside, arbutin (Martin et al., 1983; Jalal et al., 1982). On ingestion by ruminant herbivores, phenolic glycosides are readily hydrolyzed in the rumen by glycosidase enzymes (Scheline, 1991), allowing the phenolic and sugar moieties to act independently. Therefore, it may be hypothesized that any negative effects on rumen microorganisms by a phenolic compound ingested in the form of a glycoside may be accompanied by the sugar moiety providing a readily fermentable substrate. Inclusion of both quinol and arbutin in this study permits quantification of these effects.

METHODS AND MATERIALS

The *in vitro* system used in this study was the consecutive batch culture technique of Theodorou et al. (1984, 1987), which reproduces a rumen-like fermentation in the laboratory with the microbial population being maintained in the growing phase. Rumen microbial activity was assessed by gas production, volatile fatty acid (VFA) production, and percentage dry matter (DM) loss of substrate.

One hundred milligrams of dried timothy hay (*Phleum pratense*), milled to pass a 1-mm sieve, was weighed into 48 glass culture tubes (16 × 125 mm Hungate tubes, Bellco Glass Inc., Vineland, New Jersey). The tubes were arranged as two replicate series of six tubes for each of the four treatments—orcinol, quinol, arbutin (Figure 1), or control, to which no phenolic was added. The phenolic compounds were dissolved in methanol (99%) at concentrations such that additions of 0.5 ml to the hay gave the equivalent of a 10 mM solution of phenolic compounds once the methanol had been evaporated and the culture medium (9 ml) and the rumen inoculum (2 ml) had been added. The control tubes were treated with 0.5 ml of methanol, which was evaporated to dryness

under a gentle stream of air as for the phenolic treatments. Rumen liquor was collected by aspiration from a rumen-fistulated sheep fed a diet of hay and grass pellets. The rumen liquor was filtered through two layers of muslin and stored under carbon dioxide before inoculation of the tubes. At the start of the experiment, 2 ml of rumen liquor and 9 ml of anaerobic culture medium (Hungate and Stack, 1982) were inoculated into the first tubes of each series. The tubes were sealed by septum stoppers retained by screw caps; the contents of the tubes were then mixed and incubated at 38°C for 48 hr. Consecutive batch culture was achieved by transferring 2 ml of inoculum from these tubes every 48 hr into the subsequent fresh tubes in the series, containing 9 ml of Hungate and Stack culture medium plus the appropriately treated hay substrate. This was continued over a period of 12 days in which there were five transfers on days 2, 4, 6, 8, and 10. Anaerobiosis was maintained throughout by conducting all procedures under a continuous flow of oxygen-free carbon dioxide.

After each 48-hr incubation, measurements of gas production, dry matter loss, and VFA concentration were made. Gas production was measured by piercing the rubber septum in the top of the tubes with a 2-ml glass syringe and measuring the displacement of the plunger. Following incubations, the tubes were centrifuged for 8 min at 12,000 rpm. The residue left in the tubes was washed twice with distilled water before being freeze-dried and weighed to determine percentage dry matter loss. The supernatant was stored at −20°C prior to analysis for VFA concentration. To 1 ml of the supernatant, 0.25 ml of internal standard (20 mM solution of 2-ethylbutyric acid in 20% H_3PO_4) was added. Gas–liquid chromatography (GLC) analysis for VFAs was carried out on a Pye Unicam PU 4500 gas chromatography (GC) with a glass column length of 1.5 m and diameter 4 mm), packed with SP-1200 (Supelco). A sample of 0.2–0.4 μl was injected into the column at an initial injector temperature of 150°C. Helium was the carrier gas, with a flow rate of 35 ml/min and oven temperature of 140°C. Detection was by flame ionization and calibration was carried out using a mixture of VFAs (C2–C5) in approximately the same proportions and concentrations as found in the rumen samples.

Statistical analysis was carried out using the GENSTAT 5 statistical package (Lawes Agricultural Trust, Release 3.1). Because of the sequential nature of the transfers of inoculum, there was a strong possibility of carryover effects between days of measurement within each series of tubes. The statistical analysis hence examined the effects of treatments and days in a two-way factorial analysis of variance, which was blocked within days, which in turn was blocked within series of tubes. Specific comparisons were made using the LSD test (Snedecor and Cochran, 1982). Although all data are presented, the statistical analysis was conducted after omitting the results from day 2 since the composition of this first set of tubes incubated was different to subsequent batches as it contained

fresh rumen liquor. All quoted standard errors of difference (SED) derive from analyses on these restricted data.

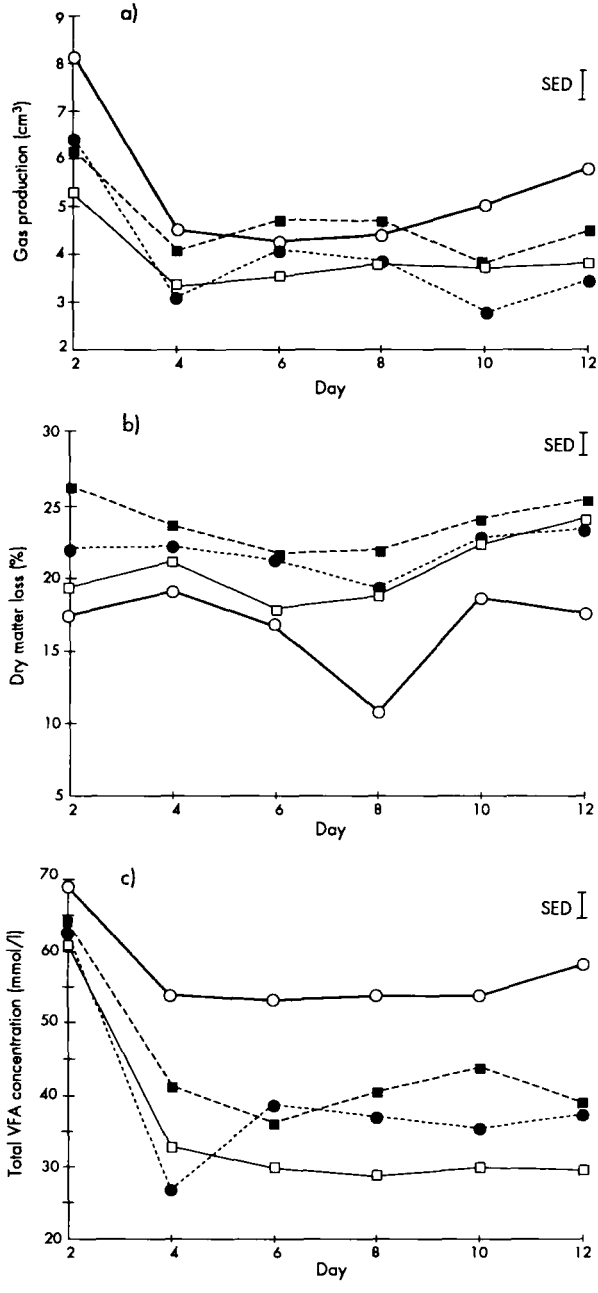
RESULTS

The presence of phenolic compounds had a significant ($F = 9.93$, $df = 3, 15$, $P < 0.001$) effect on gas production (Figure 2a). Mean gas production in the presence of orcinol (3.62 cm^3, SE 0.095) and quinol (3.44 cm^3, SE 0.226) was significantly lower ($P < 0.05$, LSD test) than the control treatment (4.25 cm^3, SE 0.120). In the tubes containing arbutin there was no overall significant effect on microbial gas production (4.75 cm^3, SE 0.299) compared to the control treatment, but gas production was significantly greater than the quinol treatment ($P < 0.05$, LSD test).

Dry matter (DM) loss of grass hay during continuous batch culture varied significantly between phenolic treatments ($F = 23.87$, $df = 3, 15$, $P < 0.001$; Figure 2b). The mean DM loss was significantly lower ($P < 0.01$, LSD test) in the presence of arbutin (16.4%, SE 1.18) and orcinol (20.5%, SE 0.72) compared to the control treatment (22.9%, SE 0.70). The addition of quinol had no significant effect on DM loss (21.2% SE 0.92) compared to the controls ($P < 0.1$). The DM loss was significantly lower in the arbutin treatment than the quinol treatment ($P < 0.001$).

There was a significant effect ($F = 80.02$, $df = 3, 15$, $P < 0.001$) of phenolic compounds on total VFA concentration (Figure 2c). Total VFA concentration was significantly higher ($P < 0.01$, LSD test) in the presence of arbutin (54.8 mmol/liter, SE 1.02) compared to the control (39.6 mmol/liter, SE 1.01). In contrast, the presence of both orcinol and quinol resulted in significantly lower total VFA concentrations compared with controls (orcinol: 30.2 mmol/liter, SE 0.96, $P < 0.01$, LSD test; quinol: 34.9 mmol/liter, SE 1.90, $P < 0.05$, LSD test). This suggests that arbutin was stimulating VFA production and orcinol and quinol were suppressing it. There were no significant phenolic treatment × day interaction effects on gas production, dry matter loss, or VFA concentration, indicating that any effects of phenolic treatment were consistent across days.

Table 1 shows the effects of phenolic compounds on acetate, propionate, and butyrate concentration. There was a significant effect of treatments on mean concentrations of acetate ($F = 18.90$, $df = 3, 15$, $P < 0.001$), propionate ($F = 116.24$, $df = 3, 15$, $P < 0.001$) and butyrate ($F = 84.91$, $df = 3, 15$, $P < 0.001$). The presence of arbutin led to a higher acetate concentration than controls ($P < 0.05$, LSD test), whereas orcinol and quinol resulted in lower acetate concentrations than the controls ($P < 0.05$, LSD test). As with acetate


a)
SED
Gas production (cm³)
Day
b)
SED
Dry matter loss (%)
Day
c)
SED
Total VFA concentration (mmol/l)
Day

concentration, the presence of arbutin resulted in a higher propionate concentration, whereas orcinol and quinol decreased the propionate concentration compared to controls (all comparisons: $P < 0.001$, LSD test). In contrast, all three phenolics, orcinol, quinol, and arbutin, resulted in a significantly higher mean butyrate concentration in comparison to the control ($P < 0.001$, LSD test). There was a significant effect of days on the concentration of butyrate ($F = 12.02$, $df = 4, 4$, $P < 0.05$), although no consistent trends were apparent (Table 1).

Incorporation of data from day 2 into the analyses led to significant ($P < 0.05$) main effects of day on all variables, thus confirming the magnitude of this methodological effect.

DISCUSSION

This study was conducted to determine whether simple phenolic compounds found in heather had an adverse effect on rumen microbial activity. Such adverse effects on microbial activity would result in reduced digestibility of the plant material and reduced supply of energy to the animal.

The presence of orcinol at a concentration of 10 mM resulted in a reduction in microbial gas production, percentage DM loss, and total VFA concentration, suggesting a toxic effect on rumen microorganisms. The nutritional significance of this reduction in the digestibility of plant material is the lower microbial production of VFAs, the fermentation products that are a major energy source for ruminants and other herbivores utilizing microbial fermentation (Van Soest, 1983). Gas production and total VFA concentrations were reduced in the presence of quinol but percentage DM loss was unaffected. The presence of arbutin, which is a glucoside of quinol, resulted in a significant reduction in percentage DM loss. This is likely to result from the release of glucose from the glucoside, as glucose has been shown to cause catabolite repression of cellulolysis by some rumen microorganisms (Morrison et al., 1990). Thus it appears that the microorganisms utilized the glucose derived from arbutin as a fermentation substrate, resulting in reduced utilization of the hay, but permitting increased total VFA and/or gas production. The arbutin added to the tubes was equivalent to the addition of 0.11 mmol of both glucose and quinol, in the form of the glucoside.

FIG. 2. The effect of additions of the phenolics orcinol (—□—), quinol (---●---), and arbutin (—○—), and solvent-only controls (--■--), on (a) gas production, (b) dry matter disappearance, and (c) volatile fatty acid (VFA) concentration in consecutive batch culture. SED shown is for comparisons of phenolic treatments within days. Unlike letters denote significant differences ($P < 0.05$, LSD test) between means pooled across days 4–12 (see text).

TABLE 1. EFFECT OF SIMPLE PHENOLIC COMPOUNDS ON ACETATE, PROPIONATE, AND BUTYRATE CONCENTRATION (MILLIMOLES PER LITER) IN CONSECUTIVE BATCH CULTURE[a]

	Acetate				Propionate				Butyrate			
Day	Control	Orcinol	Quinol	Arbutin	Control	Orcinol	Quinol	Arbutin	Control	Orcinol	Quinol	Arbutin
2	41.56	39.75	42.35	40.15	14.65	13.95	14.30	19.80	6.45	5.30	5.10	7.25
4	25.30	21.00	18.25	27.35	11.40	6.60	5.70	15.40	2.85	3.65	1.70	7.90
6	21.60	16.60	21.40	24.05	9.95	5.15	10.35	18.10	2.65	5.55	4.40	6.55
8	21.60	15.30	19.50	25.90	13.35	5.10	8.40	18.90	3.10	5.85	6.05	5.45
10	23.30	17.40	18.95	24.50	16.05	5.40	8.65	20.85	2.45	5.00	5.25	4.90
12	23.60	23.35	24.65	29.10	10.90	2.35	6.65	18.75	2.60	2.70	4.85	6.55
Mean	23.08a	18.73b	20.55b	26.18c	12.30a	4.92b	7.95c	18.40d	2.73a	4.55b	4.45b	6.55c
	SED_p	1.053	SED_w:	12.352	SED_p:	0.768	SED_w:	1.717	SED_p:	0.222	SED_w:	0.496

[a] SED_p: standard error of difference for comparisons between means of phenolic treatments pooled across days; SED_w: standard error of difference between phenolic treatments within days. Values followed by different letters indicate significant differences ($P < 0.05$, LSD test) between means pooled across days 4–12 (see text).

Assuming that microbial fermentation of 3 mol of glucose yields 4 mol of gas (Van Soest, 1983), and 1 mol of gas occupies 24.5 liters at 25°C, then complete fermentation of the glucose would have yielded 3.58 ml of gas at 25°C. The difference in gas production between the arbutin (quinol glucoside) and the quinol-only treatment was 1.30 ml. This is less than the expected maximum amount of 3.58 ml that could have been formed from the additional hexose, suggesting either incomplete hydrolysis of the arbutin by glycosidases, incomplete fermentation of the liberated glucose, or glucose-induced catabolite repression of cellulolysis, leading to fewer hexose intermediates.

The concentrations of phenolic compounds used in this experiment would be higher than the overall concentration of those individual phenolic compounds found in rumen fluid (Jung et al., 1983), although the amounts added are probably not dissimilar to the total concentration of the non-tannin phenolic compounds, about 1% of the substrate dry matter, that would be found in *Calluna* (Jalal et al., 1982). However, it has been suggested that rumen microorganisms *in vivo* would encounter effectively higher levels of phenolic compounds in the microenvironment within the cell matrix as they act on the surfaces of the plant material near the site of release of the phenolics (Chesson et al., 1982; Borneman et al., 1986). Previous studies demonstrated differential effects of the phenolics on the components of the microbial population (Hungate, 1966); particular species of bacteria (Chesson et al., 1982) or taxonomic groups of microorganisms such as the fungi (Akin and Rigsby, 1985) may not be equally susceptible to the effects of specific phenolics. This factor may have led to the differences in the observed concentrations and proportions of VFAs produced in the presence of the different phenolics; fermentation in the presence of arbutin produced contrasting patterns of VFAs compared to orcinol and quinol, which depressed acetate and propionate concentrations relative to controls but stimulated butyrate production. The general stimulation of VFA production by arbutin was consistent with an overall stimulatory effect due to its glucose component (Van Soest, 1983).

Calluna is rich in both tannin and non-tannin phenolics (Iason et al., 1993; Jalal et al., 1982). Tannin phenolics are one of the factors affecting digestibility and, hence, intake and diet selection by ruminants (Robbins et al., 1987; McArthur et al., 1993). This study has shown that simple phenolic compounds of heather—orcinol and quinol—also have negative effects on *in vitro* digestion by rumen microorganisms and are thus also likely to influence feeding ecology. However, the presence of these compounds in plants, mainly as glycosides (Jalal et al., 1982), means that on ingestion by ruminants, their negative digestive effects would be reduced or offset by the positive effects of the sugar unit that is liberated and used as a fermentation substrate. Although this effect may reduce the influence of the phenolic glycosides on digestibility, even small residual

changes in digestibility can have large population consequences for ruminants (White, 1983). The non-tannin phenolics, including orcinol and quinol, have also been hypothesized to affect intake and diet selection by deer via toxic effects following absorption (McArthur et al., 1993; Iason and Murray, 1996). However, toxic effects of specific compounds appear not to be a prerequisite for deterrency to a specialist insect herbivore (Bernays, 1990).

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OLEFINIC ACETATES, Δ-9,11-14:OAc AND Δ-7,9-12:OAc USED AS SEX PHEROMONE COMPONENTS IN THREE GEOMETRID MOTHS, *Idaea aversata*, *I. straminata*, AND *I. biselata* (Geometridae, Lepidoptera)

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Abstract—Pheromone compounds so far identified from most geometrid moths consist of all-*Z* diene, triene, or tetraene hydrocarbons with chain lengths of C_{17} to C_{21}, and their monoepoxide derivatives biosynthesized from linoleic and linolenic acids. The present study reports the occurrence of olefinic acetates as sex pheromones in three species of Geometridae. (*Z*,*Z*)-9,11-tetradecadienyl acetate and (*Z*,*Z*)-7,9-dodecadienyl acetate found in female gland extracts of *Idaea aversata* elicited significant responses from conspecific male antennae in gas chromatography with electroantennographic detection (GC-EAD). In extracts of *I. straminata*, (*Z*,*E*)-7,9-dodecadienyl acetate, (*E*,*Z*)-7,9-dodecadienyl acetate, and (*Z*,*Z*)-7,9-dodecadienyl acetate were found, and the synthetic compounds elicited strong responses from conspecific male antennae. In the third species, *I. biselata*, only (*Z*,*Z*)-7,9-dodecadienyl acetate was found in the female extracts, and this compound elicited a strong EAD

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response from the conspecific male antenna. The identities of the pheromone components in *I. aversata* and *I. straminata* were further confirmed according to their characteristic ions after GC-MS analyses. Single sensillum recordings from *I. aversata* showed two types of pheromone-detecting sensilla present on the male antenna. One type contained two receptor neurons, one of which was specifically tuned to (*Z*,*Z*)-9,11-tetradecadienyl acetate, the other to (*Z*,*E*)-9,11-tetradecadienyl acetate. A second type contained one neuron responding to (*Z*,*Z*)-7,9-dodecadienyl acetate. The two types were clearly different also with respect to external morphology, the former being considerably longer and having a larger base diameter. Also in *I. straminata* two physiological types of sensilla could be distinguished. One type contained two neurons, one of which responded to (*Z*,*Z*)-7,9-dodecadienyl acetate, the other to (*Z*,*E*)-9,11-tetradecadienyl acetate. The second type contained one neuron, responding to (*Z*,*Z*)-7,9-dodecadienyl acetate. No correlation between external morphology and physiological response of the investigated sensilla was observed in *I. straminata*. In field tests, a two-component blend containing (*Z*,*Z*)-9,11-tetradecadienyl acetate and (*Z*,*Z*)-7,9-dodecadienyl acetate in a ratio of 10 : 1 was attractive to males of *I. aversata*. This two-component blend was also attractive to males of *I. straminata*, but in a ratio of 1 : 1. High numbers of male *I. biselata* were caught in traps baited with (*Z*,*Z*)-7,9-dodecadienyl acetate alone. The incorporation of deuterium labels into pheromone components after topical application of deuterium-labeled palmitic acid confirmed that the pheromone components of *I. aversata* could be synthesized from this precursor, as has been previously observed for acetate pheromone components of many other moth species. Our results suggest that an evolutionary reversal back to the production of palmitic acid-derived pheromone components has occurred within the geometrid subfamily Sterrhinae.

Key Words—Sex pheromone, *Idaea aversata*, *Idaea straminata*, *Idaea biselata*, (*Z*,*Z*)-9,11-tetradecadienyl acetate, (*Z*,*Z*)-7,9-dodecadienyl acetate, (*Z*,*E*)-7,9-dodecadienyl acetate, Lepidoptera, Geometridae, electroantennography, single cell recording, biosynthesis, phylogeny, evolution.

INTRODUCTION

The majority of pheromones of higher Lepidoptera consist of unsaturated acetates, alcohols, or aldehydes, and the carbon skeletons of these pheromone components can be biosynthetically derived from palmitic acid (hexadecanoic acid) or stearic acid (octadecanoic acid) by a combination of chain-shortening, desaturation, reduction, and acetylation reactions (Bjostad et al., 1987). A less common type of moth pheromone component includes unsaturated hydrocarbons and their epoxides, which can be derived from linoleic and linolenic acid (Rule and Roelofs, 1989). Such pheromone components occur mainly within the superfamilies Noctuoidea and Geometroidea (Arn et al., 1992). In Geometridae, sex pheromones have been identified for around 28 species, and sex attractants have been reported for an additional 55 species (Arn et al., 1992 and references therein). In most of these reports, sex pheromones and sex attractants were

all-*Z* diene, triene, or tetraene hydrocarbons with chain lengths from C_{17} to C_{21} and/or their corresponding monoepoxides. Three hemlock loopers utilize branched hydrocarbons as their pheromones (Gries et al., 1991, 1993a,b), and a few reports indicate that olefinic acetates and aldehydes can also be used as pheromones or attractants in some species of Geometridae, but only within the subfamily Sterrhinae. For example, (*E*,*Z*)-7,9-dodecadienyl acetate (*E*7,*Z*9–12:OAc) was reported to be attractive to males of *Idaea biselata* (Biwer et al., 1975), and compounds such as (*E*,*Z*)-7,9-dodecadienal (*E*7,*Z*9–12:Ald) and (*Z*)-7-dodecenyl acetate (*Z*7–12:OAc) have also been shown to be attractive for males of this species (E. Priesner personal communication and Ando et al., 1987). *Z*7–12:OAc, *E*7,*Z*9–12:OAc, and (*E*,*Z*)-7,9-dodecadienol (*E*7,*Z*9–12:OH) were reported as attractants for males of *I. imbecilla*, *I. rubraria*, and *I. trisetata*, respectively (Szöcs et al., 1987; Ando et al., 1977, 1987).

The type of sex pheromone components used in moths may be of taxonomic significance at the genus, subfamily, and family levels, and pheromone characteristics may be used for phylogenetic analysis of different taxa (Roelofs and Brown, 1982; Löfstedt et al., 1991; Löfstedt and Kozlov, 1996). The possibility that acetates are used as pheromone components in one geometrid subfamily, whereas other subfamilies appear to use branched hydrocarbons or unsaturated hydrocarbons and/or their corresponding epoxides as pheromone components, is intriguing and deserves to be studied in more detail. In the present study we confirm the occurrence of acetates as sex pheromone components in three geometrid moths, *I. aversata* (L., 1758), *I. straminata* (Borkhausen, 1794), and *I. biselata* (Hufnagel, 1767). Active components were detected by gas chromatography with electroantennographic detection (GC-EAD) and identified by gas chromatography and gas chromatography–mass spectrometry. The activity of synthetic pheromone components was confirmed by single sensillum recordings on male antennae and by field trapping.

METHODS AND MATERIALS

Insects. Female adults of all three investigated species were collected at various sites in southern and south-central Sweden. Laboratory cultures were established from eggs laid by these collected insects. The larvae were reared on withered moist leaves from a number of plant species, predominantly willows (*Salix* spp.). The cultures were kept under a 16 L:18 D photoperiod at a temperature of 22°C and a relative humidity of 60%. Sex was determined at the pupal stage, and the emerging adults were kept individually in small cups under the same environmental conditions as the larvae.

Pheromone Gland Extraction. Females of all species started calling 3–4 h after darkness on the second day after emergence. The pheromone glands of calling females were excised and extracted for 30 min in redistilled hexane.

Electrophysiological and Chemical Analyses. For electroantennographic (EAG) recordings, excised male antennae were placed with the base in a capillary electrode filled with Beadle-Ephrussi Ringer, and grounded via an Ag–AgCl wire. The distal tip of the antenna was cut, and the exposed surface 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.

Single sensillum recordings were performed utilizing the tip-cutting technique. Either excised male antennae or intact insects were used. Excised antennae were placed with the base in a capillary electrode as described above. Intact insects were restrained in holders cut from plastic disposable pipet tips, and the head and antennae were fixed in position with dental wax. A thin tungsten wire serving as ground electrode was inserted into the abdomen. Olfactory sensilla were cut by means of two glass knives, and a recording electrode was placed in contact with the cut surface of the sensillum. The recording electrode was a glass pipet with a tip diameter of about 4 μm, filled with Beadle-Ephrussi Ringer. The recording electrode was connected to a high-impedance AC amplifier via an Ag–AgCl wire. The recorded data was analyzed on a Macintosh computer with a Maclab analog/digital interface (M. C. Macknight, Analog Digital Instruments).

In both EAG and single sensillum experiments, the antenna was continuously flushed with charcoal-filtered and moistened air (0.5 m/sec) delivered through a glass tube (8 mm ID) ending 10 mm in front of the preparation. Stimulus cartridges consisted of Pasteur pipets containing a piece of filter paper (7 × 15 mm) on which the stimulus had been applied. In the dose–response tests, serial dilutions (10^{-2}–10^{2} μg in decadic steps) of the compounds dissolved in 10 μl of hexane were applied. Air (2 ml) was passed over the filter paper in the stimulus cartridge during 0.5 sec and injected into the airstream 150 mm upstream of the antenna.

A Hewlett Packard 5830 gas chromatograph (GC) equipped with a DB-wax column (30 m × 0.25 mm ID, J & W Scientific, Folsom, California) and an effluent split, allowed simultaneous flame ionization (FID) and electroantennographic detection (EAD) of the separated pheromone components. Hydrogen was used as carrier gas, and the effluent split ratio was approximately 1 : 1. Samples were injected in splitless mode. 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 3 min following the injection and then linearly increased to 230°C at 10°C/min. The outlet for the EAD was placed in a purified airstream flowing over the antennal preparation at a speed of 0.5 m/sec. Either female pheromone gland extracts or synthetic standard were injected into the column for the analysis. GC retention times of EAD-active components were converted to equivalent chain lengths (ECLs) relative to the retention times of

homologous, straight-chain acetates (7–22:OAc). The electrophysiological methods were identical to those described above.

Individual female pheromone gland extracts were also analyzed on a Hewlett-Packard 5880 GC equipped with the same column and under the same operating conditions as described above.

Mass spectrometry with electron impact ionization (70 eV) was performed on a HP 5970B GC-MS system equipped with a 59970B computer system, and interfaced with a HP 5890 GC. Gas chromatographic conditions for analyses were the same as those described above.

Biosynthesis Experiment. [16,16,16-$^2H_3$]hexadecanoic acid (D_3-16:COOH) (Larodan Fine Chemicals, Malmö, Sweden) dissolved in DMSO (4 μg/0.2 μl) was applied topically to the pheromone glands of 2- to 3-day-old female *I. aversata* with a 10-μl syringe. After 1 hr incubation, the pheromone glands were dissected and extracted for 30 min in 6 μl redistilled hexane containing 1 ng Z8–13:OAc as an internal standard. Deuterium-label incorporation from different precursors into pheromone components was determined by GC-MS in the selected ion monitoring mode. An acquisition program was designed in which diagnostic ions (DI) of unlabeled and D_3-labeled (DI+3) pheromone acetates were monitored simultaneously and the selected ions were changed at the appropriate time during the separation based on the retention times of the synthetic standards. This allowed selective detection of each of the pheromone components and their respective D_3-labeled analogs. Thus, *m/z* 164.20 and 167.20 were chosen for detection of unlabeled and deuterium-labeled Δ-7,9–12:OAc, respectively, whereas Δ-9,11–14:OAc was monitored by *m/z* 192.20 and 195.20.

Field-Trapping Tests. Field tests were conducted in the Hågadal and Nåsten regions of Uppsala in south-central Sweden from the end of June to the beginning of August in 1992 and 1993. Synthetic blends were prepared in hexane. Red rubber septa (Arthur H. Thomas Co. Catalog No. 1780-J07) were used as dispensers, and the traps used were delta traps. Traps were placed in woodland habitats, where all the species under investigation occur, and were hung on the tree twigs or placed in low bushes approximately 1 m above the ground. Within a replicate ($N = 5$), the traps were set at least 2 m apart. The traps were checked and the captured males removed once a week, and trap positions within a series were randomized to minimize the effects of habitat heterogenities. In a first experiment, from June 26 to August 1, 1992, 14 different blends of pheromone components were tested at 50 μg loading on the dispenser (details in Table 1). In a second field test from July 4 to August 21, 1993, different doses and ratios of those pheromone components that had been most attractive in the field test of 1992 were tested.

Chemicals. Z9,E11–14:OAc was prepared as described by Hall et al. (1975). The product was further purified by flash column chromatography on silica gel in 2% diethyl ether in hexane to remove any traces of the corresponding

TABLE 1. MALE CAPTURES OF *Idaea* SPECIES IN TRAPS BAITED WITH DIFFERENT COMBINATIONS OF SYNTHETIC ISOMERS OF 9,11-14:OAC AND 7,9-12:OAC IN THE VICINITY OF UPPSALA, SWEDEN (1992)[a]

Bait composition (μg)						Total male captures			
Δ-9,11-14:OAc			Δ-7,9-12:OAc						
ZZ	*ZE*	*EZ*	*ZZ*	*ZE*	*EZ*	*I. averstata*	*I. straminata*	*I. biselata*	*I. emarginata*
50	25.5	3.5	5			1 c	0 b	0 b	0 b
	25.5	3.5	5			0 c	1 b	0 b	1 b
50		3.5	5			12 a	0 b	0 b	0 b
50	25.5		5			3 c	0 b	0 b	0 b
50	25.5	3.5				0 c	0 b	0 b	0 b
50			5			7 b	0 b	0 b	0 b
50		3.5				0 c	0 b	0 b	0 b
50	25.5					1 c	0 b	0 b	0 b
50			50			1 c	5 ab	0 b	8 ab
50						1 c	1 b	0 b	0 b
			50			0 c	0 b	112 a	0 b
50			50	5		0 c	5 ab	5 b	69 a
50			50		5	0 c	9 a	0 b	11 ab
50			50	50	5	0 c	8 ab	0 b	17 a
Control						0 c	0 b	0 b	0 b

[a]Catch numbers within a column followed by the same letter are not significantly different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello robust Mann-Whitney U test ($P > 0.05$), $N = 5$.

alcohol and contained 1.2% *E*9,*E*11-14:OAc after Kugelrohr distillation (100°C/0.02 mm).

*Z*9,*Z*11-14:OAc was prepared by Wittig reaction of propyl (triphenylphosphonium) bromide with potassium *t*-butoxide in tetrahydrofuran and the tetrahydropyranyl ether of (*Z*)-11-hydroxy-2-undecenal. The latter was prepared by coupling of propargyl alcohol with the tetrahydropyranyl ether of 8-bromooctan-1-ol using lithium amide in liquid ammonia, hydrogenation of the resulting acetylenic alcohol over Lindlar catalyst in ethanol at 0°C, and oxidation of the allylic alcohol with activated manganese dioxide in toluene. The aldehyde was used without further purification and gave 9,11-14:OAc isomers *ZE*:*EZ*:*ZZ*:*EE* in 6:7:87:<0.5 ratio after removal of the tetrahydropyran protecting group with *p*-toluenesulfonic acid in methanol and acetylation with acetic anhydride in pyridine. This material was further purified by liquid chromatography on silica gel impregnated with 10% silver nitrate eluted with a

gradient of diethyl ether in hexane to give a product containing *ZE*:*EZ*:*ZZ*:*EE* isomers of 9,11-14:OAc in 2.3:0.8:96.9: <0.5 ratio after Kugelrohr distillation (120°C/0.02 mm).

*Z*7,*Z*9-12:OAc was prepared by reduction of (*Z*)-9-dodecen-7-ynyl acetate (Brückner et al., 1988) with activated zinc in methanol–water (1:1) (Boland et al., 1987). At best, only 72% conversion was achieved even after 6 days at room temperature. The product, which was separated from unreacted starting material by liquid chromatography on silica gel, eluted with 5% diethyl ether in hexane to give material containing *ZE*:*EZ*:*ZZ*:*EE* isomers of 7,9-12:OAc in 0.7:0.6:98.7: <0.1 ratio after Kugelrohr distillation (90°C/0.03 mm).

(*Z*)-9-Dodecen-7-ynyl acetate was isomerized in sunlight with a catalytic quantity of iodine in hexane solution to give a mixture of the *E* and *Z* isomer in 61:39 ratio. This mixture was reduced with activated zinc as above and the resulting mixture of *Z*7,*E*9-12:OAc and *Z*7,*Z*9-12:OAc was separated by chromatography on a silver ion exchange column eluted with methanol at atmospheric pressure (cf. Houx et al., 1974). Fractions containing the *Z*7,*E*9-12:OAc only were combined to give material containing less than 0.3% of any other isomers after Kugelrohr distillation (86°C/0.01 mm).

GC analyses of the different isomers of these two conjugated acetates were conducted on a polar CP Wax 52CB column (25 m × 0.32 mm ID; Chrompack UK), helium carrier gas at 0.5 kg/cm^{-2}, oven temperature 70°C for 2 min, then programmed at 20°C/min to 120°C and then at 4°C/min to 230°C. The order of eluted synthetic compounds was *ZE*-, *EZ*-, *ZZ*-, *EE*-7,9-12:OAc and 9,11-14:OAc, respectively.

Scanning Electron Microscopy. To examine the possible correlation between the external morphology of the pheromone-sensitive sensilla and their response specificity, antennae of *I. aversata* and *I. straminata* were fixed in 70% ethanol and subsequently dehydrated in absolute ethanol, critical point-dried, mounted with epoxy glue on specimen holders, and coated with gold/palladium (Polaron E5500 high resolution sputter) before examination in a Jeol JSM-T330 scanning electron microscope, operated at 15 kV.

RESULTS

Chemical Analyses. In *Idaea aversata*, analysis of female pheromone gland extracts by GC-EAD showed that two compounds elicited strong responses from a conspecific male antenna (Figure 1B). ECLs of the two active peaks were 1390 and 1596, respectively. By comparison of these retention indices to those of synthetic compounds, these two compounds were tentatively identified as (*Z*,*Z*)-7,9-dodecadienyl acetate (*Z*7,*Z*9-12:OAc) and (*Z*,*Z*)-9,11-tetradeca-

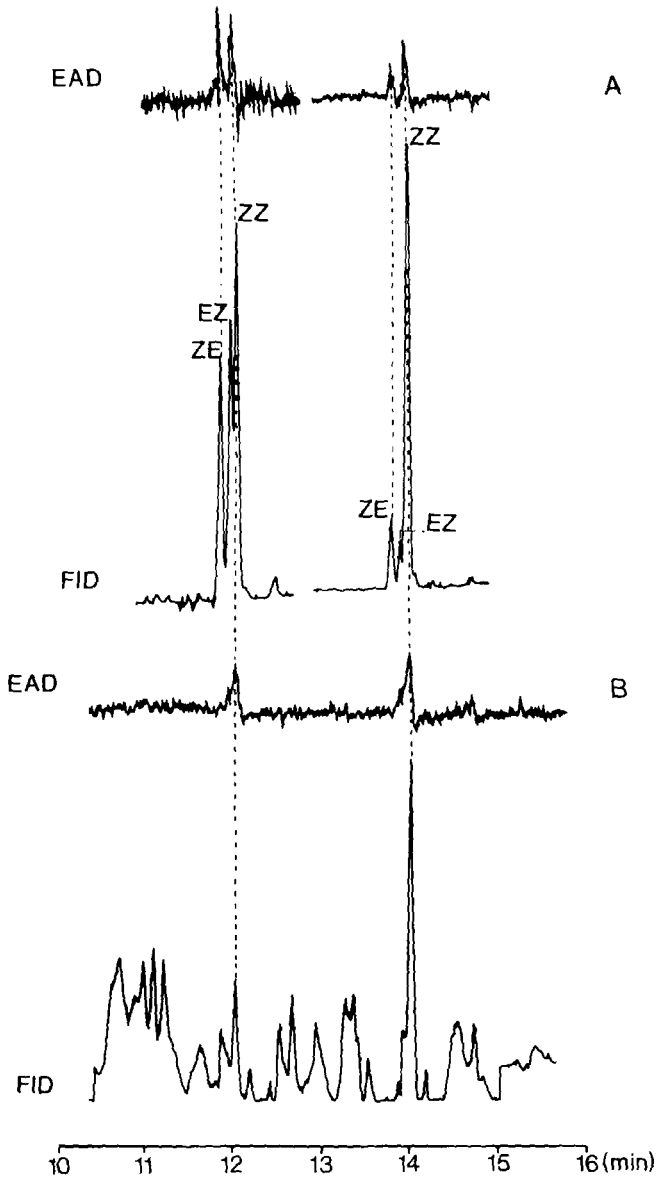

FIG. 1. GC-EAD analyses using antennae of male *Idaea aversata*. (A) Responses to the mixture of *Z*7,*E*9-, *E*7,*Z*9-, and *Z*7,*Z*9-12:OAc and the mixture of *Z*9,*E*11-*E*9,*Z*11-, and *Z*9,*Z*11-14:OAc, respectively. (B) Responses to female gland extracts.

dienyl acetate (Z9,Z11-14:OAc). Coupled GC-MS analysis of the same extracts supported the assignment of chemical structures to these two active compounds. The mass spectrum of the first eluting compound included ions at *m/z* 224 (M) and *m/z* 164 (M − 60), typical of a dodecadienyl acetate with conjugated double bonds. The spectrum of the second active peak included ions at *m/z* 252 (M) and *m/z* 192 (M − 60), indicating a tetradecadienyl acetate with conjugated double bonds. In separate GC-EAD analyses of synthetic standards, a mixture of Z7,E9-, E7,Z9-, and Z7,Z9-12:OAc produced responses to the *ZE* and *ZZ* isomers, while Z9,Z11-14:OAc containing small amounts of Z9,E11- and E9,Z11-14:OAc produced responses to the former two compounds (Figure 1A).

In *I. straminata*, combined GC-MS analyses of female pheromone gland extracts confirmed the occurrence of Z7,Z9- as well as Z7,E9- and E7,Z9-12:OAc in the females. A peak with the retention time of synthetic Z9,Z11-14:OAc was found in several gas chromatographic analyses of female extracts. When mixtures of the geometric isomers of 7,9-12:OAc and 9,11-14:OAc were tested in the GC-EAD experiments, the dodecadienyl acetates were more active than the tetradecadienyl acetates, with the Z7,Z9-12:OAc being the most active stimulus (Figure 2).

In *I. biselata*, only one active peak was found when female extracts were analyzed by GC-EAD with a conspecific male antenna as a detector (Figure 3B). The retention time of the active compound corresponded to that of synthetic Z7,Z9-12:OAc. Analysis of a synthetic sample confirmed the activity of Z7,Z9-12:OAc (Figure 3A).

Male Antennal Morphology. In *I. aversata*, sensilla trichodea of two clearly different size classes were present, as is evident from Figure 4. A large type (type I) measured ca. 70 μm in length with a diameter at the base of 3 μm. A smaller type (type II) was approximately 50 μm long and measured 2 μm at the base. The type I sensilla were much more prominent, being almost perpendicular to the antennal surface. Intermediates between the two categories were rare. In *I. straminata* the sensilla also showed a variation in size, but the differences were less pronounced.

Electrophysiology. The biological activity of the synthetic pheromone compounds was tested by EAG in all three species while single sensillum recordings were performed in two species. EAG data (Figure 5) showed that antennae of male *I. aversata* responded strongly to Z9,E11-14:OAc, Z9,Z11-14:OAc, and Z7,Z9-12:OAc. Significant responses were obtained when the male antennae of *I. straminata* and *I. biselata* were stimulated by each of the four isomers of 7,9-12:OAc. Antennae of *I. straminata* responded also to Z9,Z11-14:OAc and those of *I. biselata* showed some response to all four isomers of 9,11-14:OAc. The strongest response from male antennae of both species was elicted by Z7,Z9-12:OAc.

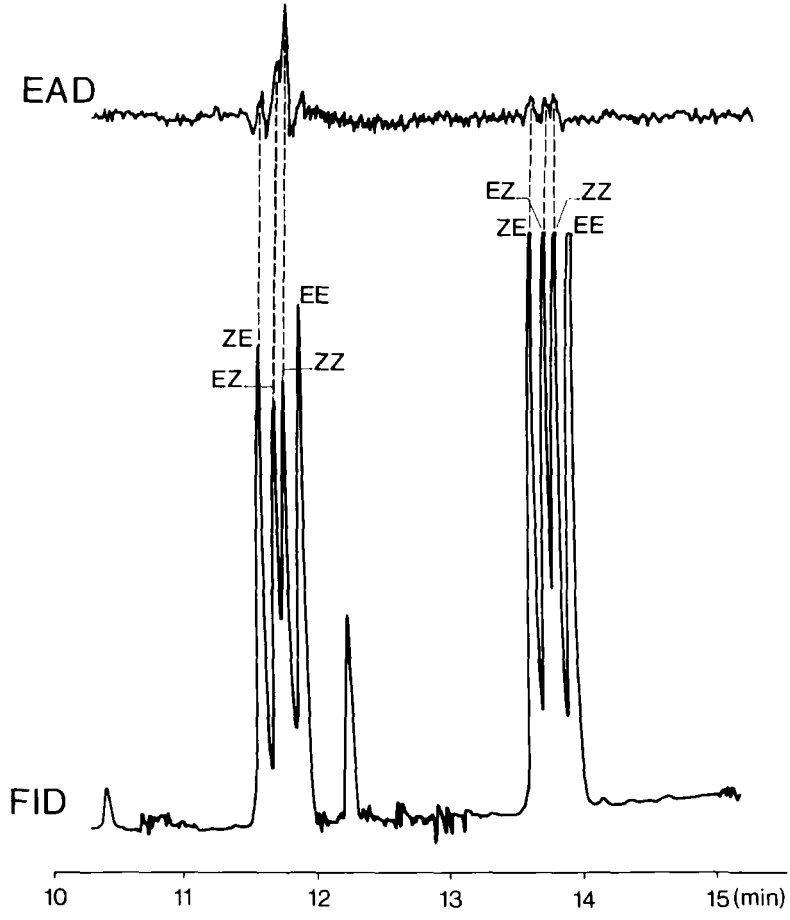

FIG. 2. GC-EAD analysis displaying male *Idaea straminata* antennal response to the mixture of four isomers of 7,9-12:OAc and four isomers of 9,11-14:OAc.

Single sensillum recordings were conducted on sensilla trichodea present on the male antennae of *I. aversata* and *I. straminata*. Recordings were made from 35 sensilla of males of *I. aversata*. All these sensilla displayed one or two receptor neurons responding to pheromone compounds. Two physiological types could be distinguished. Type I ($N = 30$) contained two neurons. One, characterized by a large action potential (spike) amplitude (neuron A), responded to *Z*9,*E*11-14:OAc, and the other, characterized by a smaller spike amplitude (neuron B) responded to *Z*9,*Z*11-14:OAc. Type II ($N = 5$) contained only one

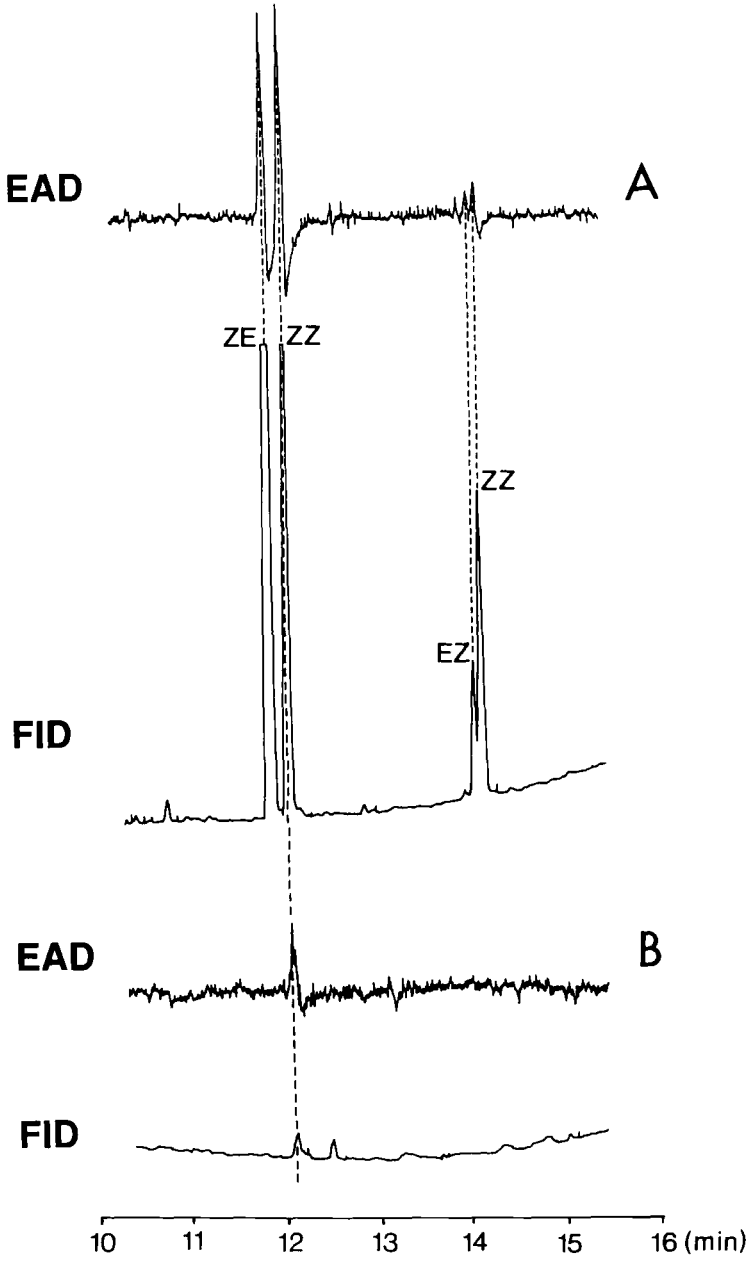

FIG. 3. GC-EAD analyses using antennae of male *Idaea biselata*. (A) Responses to the mixture of *Z*7,*E*9- and *Z*7,*Z*9–12 : OAc and *E*9,*Z*11- and *Z*9,*Z*11–14 : OAc. (B) Responses to female gland extracts.

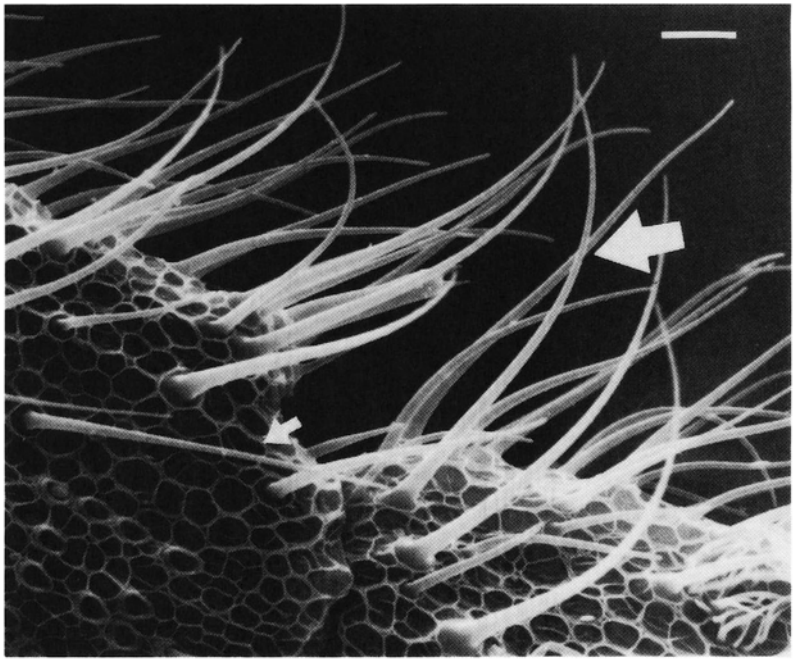

FIG. 4. The surface of male antennal segments of *Idaea aversata*, showing sensilla trichodea type I (large arrow) and type II (small arrow). Scale bar = 10 μm.

physiologically active neuron, which was specifically tuned to Z7,Z9–12:OAc. Representative recordings from these three cell types are shown in Figure 6a–c. The spike amplitudes of the two receptor neurons present in sensillum type I were quite similar, and only a thorough, computer-aided investigation of the recordings yielded an unambiguous identification (Figure 6b, inset). Type I sensilla were found among the long morphological type, while type II sensilla were found among the shorter. Dose–response tests showed that all three receptor neuron types responded to their respective key stimulus with equal sensitivity (Figure 7a–c).

In *I. straminata*, two physiologically different types of sensilla could also be distinguished. Type I (N = 17) contained two neurons, one of which responded with a large spike amplitude (neuron A) to Z7,Z9–12:OAc, and one characterized by a smaller spike amplitude responding to Z9,E11–14:OAc (neuron B). Type II (N = 13) contained one activated neuron, responding to Z7,Z9–12:OAc. Recordings from all three receptor neuron types are shown in Figure 8a–c. No correlation between external morphology and physiological specificity was established in this species. In dose–response tests, the two Z7–Z9–12:OAc

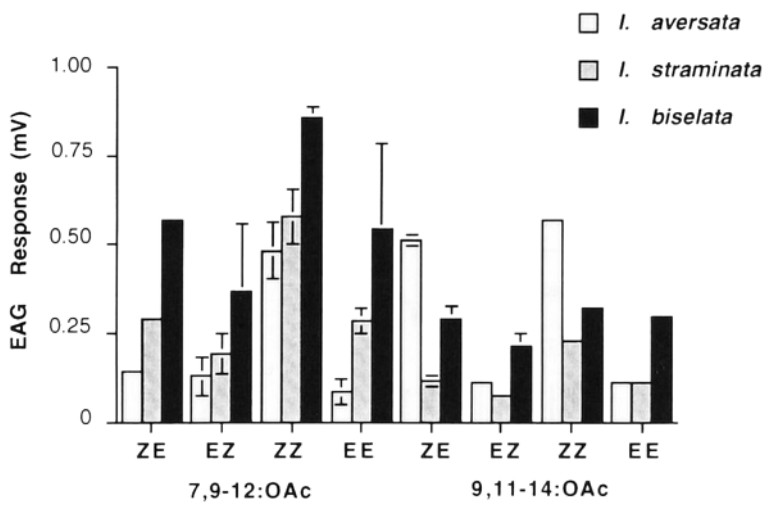

FIG. 5. EAG responses of males of *Idaea aversata*, *I. straminata*, and *I. biselata* to synthetic compounds. The blank response (air) was subtracted from each actual response. Vertical bars indicate the standard error of the mean. $N = 2$–3.

receptor neurons responded with equal sensitivity (Figure 7d). However, the *Z*9,*E*11–14:OAc receptor showed a significantly lower response.

Biosynthesis Experiments. When D_3-16:COOH was applied to the glands of *I. aversata*, there was significant incorporation into both pheromone compounds, *Z*7,*Z*9–12:OAc and *Z*9,*Z*11–14:OAc, as shown by selected ion monitoring. The D_3 analog eluted approx. 0.05 min before the unlabeled components (Löfstedt et al., 1986).

Field Tests. The results of the first field trapping test including 14 different blends of the potential pheromone compounds (Table 1), showed that males of *I. aversata* were attracted by baits containing two compounds, *Z*9,*Z*11–14:OAc and *Z*7,*Z*9–12:OAc in a ratio of 10:1, but the attraction of males was significantly increased when *E*9,*Z*11–14:OAc was included in the blend. Males of *I. straminata* were also attracted to the two component blend active to *I. aversata*, but with the compounds present in a different ratio (1:1). Such baits also trapped males of *I. emarginata*, and high captures of males of this species were obtained by adding 5 μg of *Z*7,*E*9–12:OAc to the two-component mixture. High numbers of male *I. biselata* were attracted to the bait containing only *Z*7,*Z*9–12:OAc, and adding any of the other isomers of either 9,11–14:OAc or 7,9–12:OAc reduced attractiveness significantly.

When a mixture of the two compounds *Z*9,*Z*11–14:OAc and *Z*7,*Z*9–

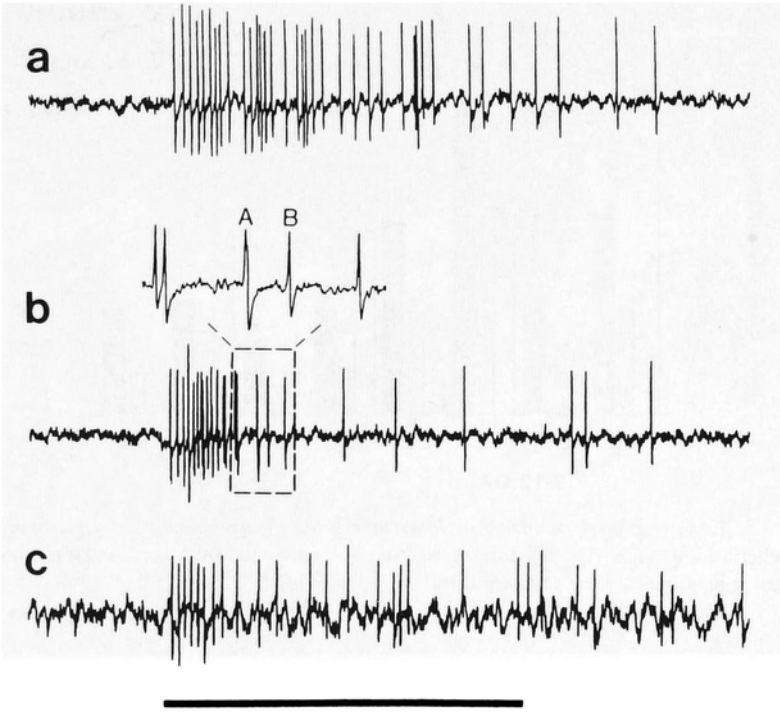

FIG. 6. Physiological responses recorded from receptor neurons present in sensilla trichodea on the male *Idaea aversata* antenna. (a) Response of the large-spike-producing neuron in the type I sensillum trichodeum to Z9,E11-14:OAc. (b) Response of the small-spike-producing neuron, present in the same sensillum type, to Z9,Z11-14:OAc. Inset shows the amplitude difference between the two receptor neurons present in sensillum trichodeum type I. (c) Response of the only receptor neuron firing in the type II sensillum trichodeum to Z7,Z9-12:OAc. Horizontal line under the records indicate duration of stimulation.

12:OAc in different ratios was tested again, the catches were low but the results confirmed those of the previous year (Table 2).

The dose-response test of Z7,Z9-12:OAc showed that *I. biselata* males responded in high numbers to baits with doses in the range of 5-500 μg. Reducing the loading further to 0.5 μg gave significantly lower catches (Table 3). No males of *I. emarginata* were trapped by baits containing 100 μg of Z7,E9-12:OAc. The attraction of males of *I. biselata* to 100 μg of Z7,Z9-12:OAc was not significantly affected by the addition of 10 μg Z7,E9-12:OAc (Table 3).

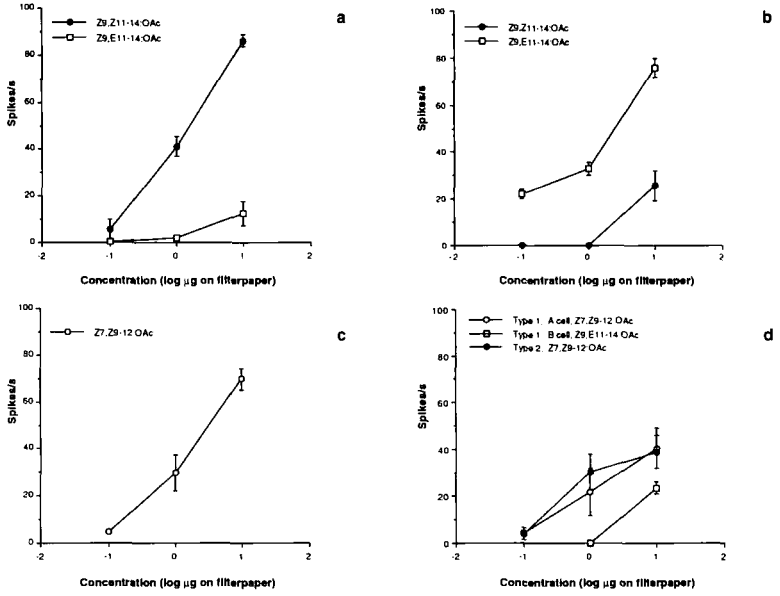

FIG. 7. Dose-response relationships for the different types of receptor neurons in *Idaea aversata* (a–c), and *I. straminata* (d). (a) *I. aversata* sensillum type I: responses of A cell to Z9,Z11-14:OAc and Z9,*E*11-14:OAc. (b) *I. aversata* sensillum type I: responses of the B cell to Z9,Z11-14:OAc and Z9,*E*11-14:OAc. (c) *I. aversata* sensillum type II: response of the single activated receptor neuron to Z7,Z9-12:OAc. (d) *I. straminata* sensillum type I and II: responses to Z7,Z9-12:OAc and Z9,Z11-14:OAc.

DISCUSSION

Our chemical analyses, electrophysiological assays, and field tests support the identification of tetradecadienyl and dodecadienyl acetates as pheromone components in the genus *Idaea*. Thus we were able to confirm previous reports on such similar compounds acting as sex attractants for certain geometrid moths (cf. Arn et al., 1992). Conjugated tetradecadienyl and dodecadienyl acetates, alcohols, and aldehydes have earlier been reported as sex pheromone components in a number of species belonging to the Pyralidae, Tortricidae, and Noctuidae families (Arn et al., 1992).

Relatively small numbers of insects were available for this work, and the identifications are by no means complete. In *I. aversata*, Z7,Z9-12:OAc and Z9,Z11-14:OAc, were identified in extracts of female glands by their GC retention times and mass spectra, and these components caused strong EAG responses in GC-EAD analyses of the female extract. In the field test, a 1:10 mixture of

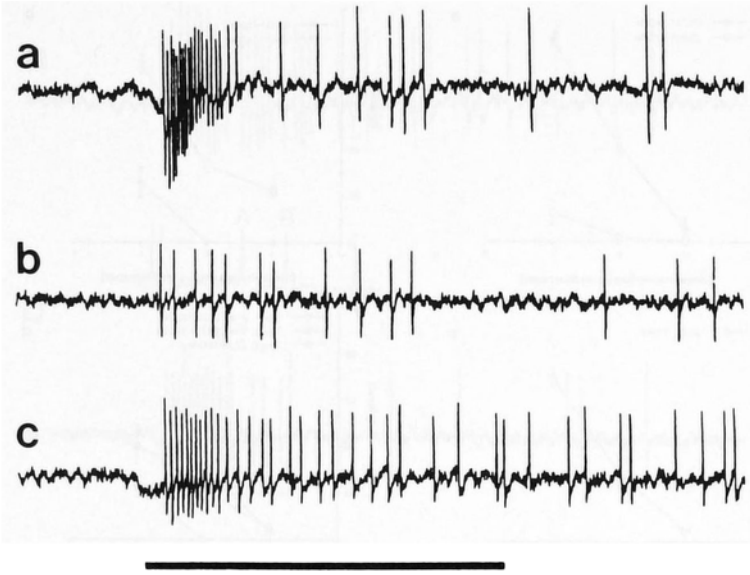

FIG. 8. Physiological responses recorded from receptor neurons present in sensilla trichodea on the male *Idaea straminata* antenna. (a) Response of the large-spike-producing neuron in the type I sensillum trichodeum when stimulated with Z9,E11-14:OAc. (b) Response of the small-spike-producing receptor neuron, present in the same sensillum type, to Z7,Z9-12:OAc. (c) A strong response was elicited by the same compound in the neuron present in the sensillum type II. Horizontal line under the records indicate duration of stimulation.

TABLE 2. MALE CAPTURES OF TWO *Idaea* SPECIES IN TRAPS BAITED WITH DIFFERENT RATIOS OF SYNTHETIC COMPOUNDS, Z9,Z11-14:OAC AND Z7,Z9-12:OAC IN THE VICINITY OF UPPSALA, SWEDEN (1993)[a]

		Total male captures	
Z9,Z11-14:OAc (μg)	Z7,Z9-12:OAc (μg)	*I. aversata*	*I. straminata*
50	0.5	8 a	0 b
50	5	10 a	1 b
50	25	5 a	0 b
50	50	0 b	5 a

[a]Catch numbers in each column followed by the same letter are not significantly different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello robust Mann-Whitney U test ($P > 0.05$), $N = 5$.

TABLE 3. MALE CAPTURES OF *Idaea biselata* IN TRAPS BAITED WITH DIFFERENT DOSES AND DIFFERENT RATIOS OF SYNTHETIC COMPOUNDS, *Z*7,*E*9–12:OAc AND *Z*7,*Z*9–12:OAc IN THE VICINITY OF UPPSALA, SWEDEN (1993)[a]

*Z*7,*E*9–12:OAc (μg)	*Z*7,*Z*9–12:OAc (μg)	Total male catches
	500	101 a
	50	95 a
	5	93 a
	0.5	15 b
100		5 b′
	100	90 a′
10	100	116 a′

[a]Catch numbers followed by the same letter are not significantly different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello robust Mann-Whitney U test ($P > 0.05$), $N = 5$.

these two compounds was attractive to male moths, and their attractiveness seemed to be increased by addition of *E*9,*Z*11–14:OAc. For *I. straminata*, the presence of *Z*7,*Z*9–12:OAc in female pheromone gland extracts was confirmed by GC-MS, and a peak at the retention time of *Z*9,*Z*11–14:OAc was observed in GC analyses. *Z*7,*Z*9–12:OAc was the most active of the isomers of 7,9–12:OAc and 9,11–14:OAc in GC-EAD analyses, and in field tests a 1:1 mixture of *Z*7,*Z*9–12:OAc and *Z*9,*Z*11–14:OAc attracted male moths. Biwer et al. (1975) and Szöcs et al. (1987) reported that males of *I. biselata* are attracted to *E*7,*Z*9–12:OAc and according to E. Priesner (personal communication referred to in Arn et al. 1992), *Z*7–12:OAc is also attractive to this species. In the present study, GC-EAD analyses showed that both *Z*7,*E*9–12:OAc and *Z*7,*Z*9–12:OAc elicited strong male antennal responses, but only the latter compound was identified from female extracts. *Z*7,*Z*9–12:OAc alone attracted a high number of male moths in the field, and no increase of male catches was observed after addition of *Z*7,*E*9–12:OAc. Since *E*7,*Z*9–12:OAc was not found in female extracts of *I. biselata*, this compound was not tested in our field tests.

The overall catches of male *I. aversata* and *I. straminata* were rather low, although the chemical analyses and electrophysiological data from EAG, GC-EAD, and single sensillum recordings of both species were mutually corroborating. The low catches could be due to the poor weather during the period of field tests, especially in 1993. Missing minor components, suboptimal ratios between pheromone components, or the presence of minor impurities in the synthetic blends with antagonistic effects on male attraction may more likely have contributed to the observed low attraction, since we observed quite a few males flying around the traps. Compounds with conjugated double bonds are

known to isomerize on the rubber septa used as pheromone dispensers (Brown and McDonough, 1986). The composition of the blends released from the septa may, after a short time, deviate significantly from what was applied to the septa.

Among eight synthetic compounds tested, the suggested major pheromone components elicited the highest EAG responses in all three species assayed. The responses of male sensilla trichodea to the female sex pheromone in Lepidoptera are well known, but the single sensillum recordings reported in the present paper are the first published on geometrid male responses to female sex pheromone components such as Z9,Z11-14:OAc,Z7,Z9-12:OAc, etc. A response pattern often encountered in various moth orders was found (Den Otter, 1977; Hansen, 1984; Priesner et al., 1984; Hansson et al., 1987, 1990): a large spike amplitude cell responding to the main pheromone component in the female pheromone gland extracts and a small spike amplitude cell responding to the minor component. The B neuron in type I sensilla of male antenna of *I. aversata* has a smaller spike amplitude responding to Z9,*E*11-14:OAc, which acts as a behavioral antagonist as demonstrated in the field bioassay. Z9,*E*11-14:OAc has not been found in pheromone gland extracts of female *I. straminata*, but the presence of a receptor specific for this compound in males suggests that its behavioral activity should be further investigated.

The use of sex pheromones and sex attractants for taxonomic and phylogenetic analysis should be carried out with caution since considerable diversity exists in the placement of functional group, double-bond positions, and chain lengths of pheromone components among species of the same genus (Renou et al., 1988). Roelofs and Bjostad (1984) have postulated that the elucidation of pheromone biosynthesis is a more useful criterion than comparison of the pheromone components themselves for determining evolutionary relationship. The sex pheromones used by the great majority of lepidopteran species so far investigated consist of unsaturated straight-chain aliphatic acetates, aldehydes, or alcohols with chain lengths of 12–18 carbons. Previous research on the biosynthesis of these pheromone components has shown that they are typically synthesized de novo from acetyl-CoA and malonyl-CoA in the pheromone glands, which produce palmitic or stearic acid via the fatty acid cycle. These acids in turn serve as precursors for a number of different acyl intermediates that can be generated by various combinations of desaturation and chain shortening (Bjostad et al., 1987). Δ11-Desaturation seems to account for the largest number of unsaturated pheromone components, and this type of pheromone component is widely distributed among ditrysian moths (Löfstedt, 1991). In females of *Spodoptera littoralis*, the sex pheromone component Z9,*E*11-14:OAc is synthesized by chain-shortening of palmitic acid to tetradecanoic acid, which, by action of a *E*11 desaturase and subsequently a Z9 desaturase, is converted into (*Z*,*E*)-9,11-tetradecadienoate (Martinez et al., 1991). Reduction and acetylation of this

compound finally leads to formation of the tetradecadienyl acetate. The incorporation of deuterium-label into pheromone acetates after applying D_3-palmitic acid onto the pheromone gland of *I. aversata* shows that palmitic acid can serve as a precursor for pheromone biosynthesis in this species. However, further experiments are needed to clarify whether or not the pheromone biosynthesis of *Idaea* species involves the same pathway as that found in *S. littoralis*.

The biosynthesis of hydrocarbon pheromone components, such as those used by most species of geometrids, has only been investigated in the arctiid moth, *Phragmatobia fuliginosa* (Rule and Roelofs, 1989). Its pheromone component, (Z,Z,Z)-3,6,9-heneicosatriene is derived from chain elongation of linolenic acid to docosatrienoic acid, presumably followed by decarboxylation. Thus, biochemical evidence suggests that two very different biosynthetic pathways are used for the production of the two major compound classes used as pheromone components in Geometridae, of which the less common pathway is similar to the route employed in most other ditrysian moths.

The higher classification (phylogeny) of Geometridae is poorly resolved. Among the commonly recognized subfamilies Archiearinae, Oenochrominae (s.str.), Geometrinae, Ennominae, Larentiinae, and Sterrhinae, it is quite likely that at least the latter is polyphyletic (M. Scoble, personal communication). There is no reason to view "Sterrhinae," including *Idaea*, as the sister group to the rest of the Geometridae. The subfamily that has often been considered as the most primitive within Geometridae is the Archiearinae. Where larvae are known, the prolegs are all present (although somewhat reduced), which is unlike the typical, and characteristic, geometrid situation. However, this view is not based on any particularly detailed analysis, and Archiearinae may eventually be shown to belong within the Ennominae (M. Scoble, personal communication). There are less than a dozen species of Archierinae in the world and nothing is known on the composition of their pheromones. Ennominae include about half of the species of Geometridae. Oenochrominae are polyphyletic; true Oenochrominae are probably confined to the Australian region, and this group may, as with Archiearinae, eventually be subsumed into the Ennominae. Again the association of Geometrinae with Ennominae may be close. So the picture may be one group of Archiearinae, Oenochrominae (s.str.), Geometrinae, Ennominae; another group including the Larentiinae; and a few independent groups from the "Sterrhinae." To these groupings can be added various genera from the Oenochrominae (s.l). The phylogenetic situation is clearly involved and unresolved (M. Scoble, personal communication).

According to the relationships between the chemical structures of identified sex pheromones or sex attractants reported and the taxonomic position of species in Geometridae, Szöcs et al. (1991) drew a distribution pattern showing that methylene-interrupted polyenic hydrocarbons and their oxygenated derivatives

are predominant in Oenochrominae, Larentiinae, and Ennominae, and olefinic acetates and derivatives are used by species in Sterrinae and Geometrinae. The limited information on pheromones of Geometridae currently available can obviously not be used to resolve the phylogeny of the family. However, it is interesting to discuss the possible ancestral state of pheromones in geometrids as this could shed some light on how pheromone communication systems may change in evolutionary time. Minet (1991) suggested that the superfamily Geometroidea should include: Geometridae, Sematuridae, and Uraniidae. Pheromones of Sematuridae and Uranidae are not known. He identified Drepanoidea (i.e., Drepanidae and Epicopeiidae) as the sister group to Geometroidea. All sex attractants reported from Drepanidae are similar to the pheromones identified from *Idaea* (Arn et al., 1992). Thus, it appears likely that the common ancestor of Geometroidea and Drepanoidea possessed the ability to produce unsaturated acetate pheromone components from palmitic acid, and this type of pheromone most likely belongs to the groundplan of all ditrysian moths (Löfstedt and Kozlov, 1996). In general it is more likely that the presence of a complex apomorphic (derived) character in many entities (species, genera, families, etc.) is the result of common heritage rather than independent evolution, as parallel/convergent evolution would depend on similar functional selection pressure operating on all the taxa under consideration. Thus, we propose that the multistep biosynthetic machinery required to produce pheromone components derived from linoleic/linolenic acids has evolved once in the evolution of Macrolepidoptera, but the production of the actual pheromone components has been subsequently and repeatedly turned on and off in the evolution of certain taxa within Geometroidea (and Noctuoidea, where such compounds occur in several families such as Noctuidae, Arctiidae, and Lymantriidae). This hypothesis would be supported if the production of "the other type of pheromone component" could be induced in "nonproducing taxa" by the artificial administration of critical "missing" precursors. Finally, identification of pheromones from certain key groups among Macrolepidoptera, as well as better resolved phylogenies based on all relevant characters, are critical to allow a rigorous reconstruction of pheromone evolution in higher Lepidoptera. With respect to geometrids, the presumed primitive geometrid subfamily Archiearinae and the sister groups of Geometridae (Sematuridae and Uraniidae), for which information on pheromone composition is scanty or missing, deserve special attention.

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THE IMPORTANCE OF SEQUESTERED IRIDOID GLYCOSIDES AS A DEFENSE AGAINST AN ANT PREDATOR

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Abstract—We reared larvae of *Junonia coenia* Hubner (Nymphalidae) on artificial diets with trace concentrations of iridoid glycosides and on leaf diets with higher concentrations of iridoid glycosides. We offered these caterpillars to predacious ants and observed the effects of the following on predation: diet (artificial vs. leaf), site (ant colonies in dry vs. wet areas), instar (early vs. late), and time (changes in predation over five days). Diet and site were consistently significant predictors of the ants' propensities to reject prey and the caterpillars' abilities to escape predation. Leaf-diet caterpillars escaped more frequently than artificial-diet caterpillars, and ants from dry sites were more likely to reject prey than ants from wet sites. The percentage of iridoid glycosides found in individual caterpillars was also a good predictor of the probability of rejection by predators and prey escape. Caterpillars with higher levels of iridoids were more likely to be rejected and to escape, suggesting that sequestered iridoid glycosides are a defense against predaceous ants.

Key Words—Predation, iridoid glycosides, *Junonia coenia*, *Formica planipiles*, *Plantago lanceolata*, specialist herbivore, larval defenses, unpalatability, sequestration.

INTRODUCTION

Several recent studies have shown that some specialist insect herbivores are better protected than generalists against invertebrate predators (Bernays and Cor-

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nelius, 1989; Bernays, 1988; Stamp, 1992; Dyer and Floyd, 1993). However, it is still unknown what mechanisms are responsible for this difference. One possibility is that some specialist insects sequester plant secondary compounds that may be used as a defense (Duffey, 1980; Brower, 1984; Bowers, 1991; Dyer, 1995). Although some generalist insects can sequester plant secondary compounds (e.g., arctiids, Rothschild, 1977; Rothschild, et al., 1979), and not all specialists can sequester such compounds (Bowers, 1992), this ability may be critical in protecting many specialists against natural enemies.

The efficacy of such chemical defenses may be affected by several factors. Certain predators, for example, exhibit intraspecific phenological (Hagen et al., 1976) or spatial (Dyer and Floyd, 1993) variation in their responses to various defenses. Furthermore, other prey characteristics, such as developmental rate or body size, could either augment or weaken chemical defenses. It is important to consider confounding factors such as these when testing the overall effectiveness of a chemical defense.

In this study we examined the importance of iridoid glycosides in the larval diet of the buckeye, *Junonia coenia* Hubner (Nymphalidae), as a deterrent to the ant, *Formica rufa planipiles* Creighton (Formicidae). We also examined the effects on predation of prey developmental stage, previous predator exposure to prey, and predator habitat type.

The iridoids, aucubin and catalpol, are sequestered by buckeye caterpillars (Bowers and Collinge, 1992), presumably for defense against predators (Stamp, 1992; De la Fuente et al., 1994). These caterpillars are specialists and regularly feed on narrow-leaved plantain (*Plantago lanceolata* L.: Plantaginaceae) (Bowers, 1984) and on members of three other families of plants that contain iridoid glycosides (Bowers, 1991). Iridoid glycosides are terpene-derived compounds that are synthesized in plants by the mevalonic acid pathway (Bowers, 1991). These compounds generally are noxious to herbivores (Bowers, 1991) and are used as defenses by herbivores against predators (Nishida and Fukami, 1989). Narrow-leaved plantain contains the iridoids aucubin and catalpol (Duff et al., 1965; Bobbitt and Segebarth, 1969).

Formica planipiles is sympatric with *J. coenia* in northern California, and Dyer has observed *F. rufa*-group species preying on buckeye caterpillars. *Formica rufa*-group species can be voracious predators on a variety of prey (Skinner and Whittaker, 1981; Cherix and Bourne, 1980); one colony, for example, can harvest over 21,000 arthropod larvae in a single day (Hölldobler and Wilson, 1990).

We asked the following questions about the interactions between *F. rufa* and buckeye caterpillars that have sequestered variable levels of iridoid glycosides: (1) Are buckeye caterpillars that have sequestered high concentrations of aucubin and catalpol better protected against ant predation than caterpillars that have sequestered low concentrations of aucubin and catalpol? (2) Do buckeye

caterpillars become more unpalatable to ants as they reach later developmental stages (because of increased levels of sequestered compounds)? (3) Do ants learn to avoid unpalatable buckeye caterpillars over short periods of time? (4) Do colonies of ants with different resource availabilities show different responses to the Buckeye caterpillars?

METHODS AND MATERIALS

Three field experiments were conducted in August 1992 at the Sierra Nevada Aquatic Research Laboratory (SNARL), which is situated at 37°36'N 118°51'W in the Great Basin Desert at the base of the eastern Sierra Nevada. We used two sites at SNARL; one site was on the upper third of a glacial moraine, about 0.25 km south of SNARL, and the second site was along Convict Creek, which is at the base of the moraine and runs through SNARL. We used ants from eight colonies of *F. planipiles* as predators; for each site, we used four colonies within 50 m of each other. Before choosing colonies, we examined them to estimate subjectively the mound size (above ground volume of nest materials) and foraging activity (number of ants leaving and returning to the nest) and tried to select colonies with approximately the same sizes and foraging rates.

Larvae from eggs laid by female buckeyes were collected from Buck Meadows, California (about 80 km west of SNARL). The ants were an appropriate species of predator for these experiments because they co-occur with buckeyes in many sites near SNARL (including the Buck Meadows site). However, we used the SNARL colonies because they were naive to the prey (the host plants do not occur at SNARL), thus their reactions to caterpillars with variable amounts of iridoids would not be affected by previous encounters. Caterpillars in instars 2 and 3 were classified as early-instar caterpillars, while those in instars 4 and 5 were classified as late-instar caterpillars.

Larvae were reared on two different diets: a leaf diet consisting of fresh, new *P. lanceolata* leaves, which contain 4–8% dry weight aucubin and catalpol (Bowers and Stamp, 1993), and an artificial diet containing only trace quantities (less than 1% dry weight) of *P. lanceolata*. The plantain leaves used in the artificial diet were of all ages, including ages with lower levels of iridoid glycosides (see Bowers and Stamp, 1993). Thus the iridoid glycoside concentration of the artificial diet was approximately 1/100th that of the leaves fed to larvae, and probably even lower. The *P. lanceolata* leaves were collected from Buck Meadows, California. The artificial diet was based on a recipe by Nijhout (unpublished data) and included the following for one 53-g batch (dry weight): 2.5 g Wesson salts, 6.3 g sucrose, 16.3 g wheat germ, 8.3 g casein, 5 g yeast, 1.7 g cellulose, 340 mg cholesterol, 300 mg methyl paraben, 2 g Vanderzandt vitamin mix, 500 mg ascorbic acid, 500 mg aureomycin, 330 mg sorbic acid,

5 g dried and powdered *P. lanceolata* leaves, 4 g agar, 1 ml linseed oil, and 227 ml water.

An additional 50 caterpillars were reared on artificial or leaf diets (25 on each diet) to determine mean iridoid glycoside concentrations for caterpillars reared on these different diets. For these caterpillars, and for caterpillars used in experiment 3, iridoid glycoside content was determined using gas chromatography of the trimethyl silylated derivatives of the iridoids (Gardner and Stermitz, 1988; Bowers and Collinge, 1992). Caterpillars were starved for 24 hr, lyophilized, and ground to a fine powder for analysis.

Experiment 1. Individual buckeye caterpillars were offered to six of the eight *F. planipiles* colonies by placing them along main foraging trails and within 5 m of the colony entrance. This method was not utilized to mimic natural encounters between ants and caterpillars, but to maximize predator–prey interactions in order to test the efficacy of iridoids against the ants, which was the major goal of this study. However, the design was not entirely artificial. Foraging *F. planipiles* can encounter buckeyes on the ground along and near the foraging trails, because caterpillars often move along the ground to access new host plants and often are found on lower leaves (personal observations).

The experimental design was a full factorial, repeated over five successive days, with site (three creek colonies vs. three moraine colonies), diet (artificial vs. leaf), and instar (early vs. late) as main effects. Each cell in the experimental design contained three replicates, which were spatially (different foraging trails) or temporally (different times of day) separated. Each colony therefore received 12 caterpillars per day: three early-instar artificial-diet caterpillars, three late-instar artificial-diet caterpillars, three early-instar leaf-diet caterpillars, and three late-instar leaf-diet caterpillars.

The experiments were conducted between 06:00 and 19:30 hr, but were usually restricted to hours of greatest ant activity (07:00–10:00 and 15:00–18:00). The first colony visited each day was selected randomly, followed by a predetermined visitation sequence among the colonies. The caterpillars were offered to ant colonies by selecting them haphazardly from prepacked and marked containers; each container had the diet, colony, and instar marked. Caterpillars from different diet treatments were matched for similar size and mass. Two caterpillars (on two different foraging trails) were given to each colony per visit.

Each offering, lasting at least 5 min, began when an ant encountered a caterpillar. We recorded the following response variables: number of rejections, number of ants touching the caterpillar at the end of each minute, and whether the caterpillar escaped or was eaten. A rejection consisted of an individual ant approaching, touching, and permanently leaving a caterpillar. It was obvious when the ant was leaving the caterpillar for recruitment (rather than rejecting it), because it would walk in growing circles which always brought it back to the prey. In these cases, the individual would eventually return with other ants.

If at least one ant was already touching a caterpillar, no further rejections (i.e., another ant encountering and leaving the caterpillar) were counted. Caterpillars "escaped" when they were rejected by at least five ants or when they were not eaten within the 5-min time period. The rejection and escape variables are different because caterpillars that avoided being eaten for 5 min were not always touched and rejected by the ants. The number of caterpillars completely rejected was therefore not equal to the number of caterpillars that escaped because a caterpillar that was never touched (and therefore escaped predation) was counted as a missing value for the rejection response variable. This distinction between the two variables is important because some caterpillars were completely ignored by the ants and therefore could never be considered rejected (even though they may have escaped predation). If a caterpillar moved too far from the foraging trail before the 5 min was over, it was returned to the trail. By 5 min it usually was obvious if the ants were going to take the caterpillar back to the nest.

Rejections were pooled into three categories: no rejections, some rejections (less than five), and completely rejected (five or more); this pooling was based on preliminary studies with the ants and previous work with other species of ants (Dyer and Floyd, 1993; Dyer, 1995, unpublished data). The rejection categories and the escape response variable were dependent variables in logit models (see Christensen, 1990), which is the most appropriate method for testing associations between categorical dependent and independent variables (Feinberg, 1970; Christensen, 1990). These models allowed us to test both the main effects and interactions of the variables diet, site, day, and instar on ant response.

To test the effects of the independent variables on recruitment, the number of ants visiting a given caterpillar every minute was the dependent variable in a repeated measures analysis of variance, with time as the repeated measure and diet, site, and instar as fixed effects. To test the effects of the independent variables on possible changes in recruitment due to learning over a longer period of time, the numbers of ants visiting every minute were totaled over the 5-min period. This total was used as a dependent variable in a repeated measures analysis of variance, with day as a repeated measure and diet, site, and instar as fixed effects. All means for numbers of ants visiting are reported $\pm$ 1 SE.

Experiment 2. Because it is possible that buckeyes might be protected by automimicry (sensu Brower, 1984), we compared predation by naive ants that were offered caterpillars reared on both diets with that of naive ants that were only offered caterpillars reared on artificial diets. Caterpillars were offered to two of the eight colonies (neither of which was used for experiment 1) during a separate five-day period. The methods used were the same as those in experiment 1, with the exception of the diet treatment; the two colonies (one creek and one moraine colony) received only caterpillars reared on artificial diets. We used chi-square contingency tables to compare predation on artificial-diet caterpillars from colonies fed caterpillars reared on both diets (data from the six

colonies in experiment 1) with that from colonies fed only artificial-diet caterpillars.

Experiment 3. To examine correlations between iridoid glycoside concentrations and ant responses, we conducted a third experiment in which the caterpillars were retained for iridoid glycoside analyses. This experiment was conducted after the experiments described above were completed and was identical in design to the five-day experiments with the following exceptions: the experiment was conducted over a two-day period, only the three colonies at the creek site were used, only fifth-instar larvae were used, and the caterpillars were taken from ants before they were dragged into the nests. Caterpillars were allowed to recover for 24 hr after the experiment and were fed their original diets during this time; this recovery period permitted caterpillars to regain iridoids lost in secreted hemolymph or regurgitant. To clear the gut, the caterpillars then were starved for another 24 hr and lyophilized.

To test for correlations between levels of iridoids and ant response, the rejection categories and the escape response variable were dependent variables in logistic regressions (see Christensen, 1990) with percent aucubin and percent catalpol as predictors. The number of replicates was too small to examine relationships between iridoids and number of ants visiting the caterpillars.

RESULTS

Caterpillars reared on leaf diets had significantly higher concentrations (percentage dry weight) of iridoid [leaf-diet aucubin mean = 4.53%, artificial-diet aucubin mean = 0.36%, unequal variances $t_{(39.3)} = -7.74$, $P < 0.001$; leaf-diet catalpol mean = 3.42%, artificial-diet catalpol mean = 0.12%, unequal variances $t_{(40)} = -8.78$, $P < 0.001$]. Late-instar caterpillars had significantly higher percentages of aucubin than early instars, but there were no significant differences between instar categories in the concentrations of catalpol [late-instar aucubin mean = 3.03%, early-instar aucubin mean = 1.31%, unequal variances $t_{(23.5)} = -2.41$, $P = 0.024$; late-instar catalpol mean = 2.19%, early-instar catalpol mean = 1.08%, $t_{(67)} = -1.34$, $P = 0.184$].

Experiment 1. A total of 360 caterpillars were offered to three creek colonies and three moraine colonies. Day was never a significant predictor of the dependent variables, so it was excluded from subsequent models to keep cell sizes large enough for meaningful comparisons (see Christensen, 1990). Diet [$\chi^2_{(1)} = 25.97$, $P < 0.001$] and site [$\chi^2_{(1)} = 22.69$, $P < 0.001$] were significant predictors of the escape variable, while instar was not [likelihood ratio for unsaturated logit model: $\chi^2_{(8)} = 12.88$, $P = 0.116$]. Leaf-diet caterpillars were much more likely to escape than were artificial-diet caterpillars (Figure 1), and creek colonies were much more likely to eat caterpillars than were moraine colonies (Figure 2). None of the higher order interactions were significant.

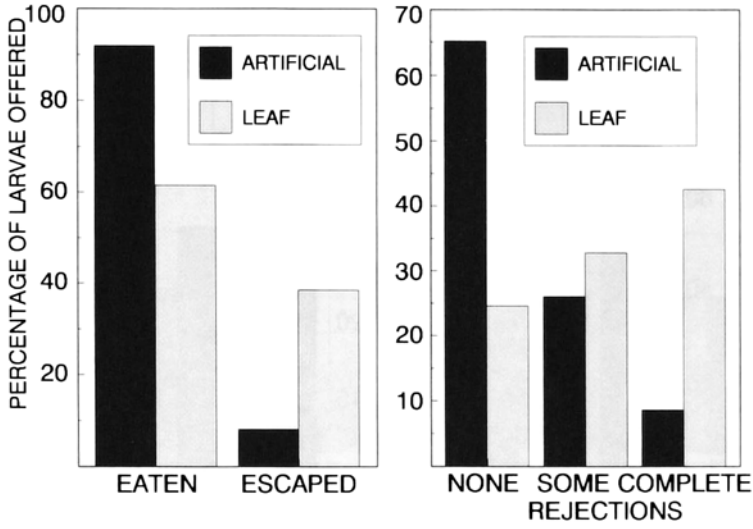

FIG. 1. The association between larval diet and predation response variables. "Leaf" refers to caterpillars reared on *Plantago lanceolata*, while "artificial" refers to caterpillars reared on an artificial diet with trace quantities of *P. lanceolata*. The *y* axes represent percentages of all larvae ($N = 360$), with specific diets that received the predator response indicated on the *x* axis. For example, 91% of the artificial-diet caterpillars offered to the ants were eaten. The predator responses on the *x* axis were categorical dependent variables in logit models.

Diet [$\chi^2_{(2)} = 44.44$, $P < 0.001$] and site [$\chi^2_{(2)} = 10.27$, $P = 0.006$] were also significant predictors of rejection, while instar was not [likelihood ratio for unsaturated logit model: $\chi^2_{(12)} = 12.64$, $P = 0.396$]. Leaf-diet caterpillars were more likely to be rejected than artificial-diet caterpillars (Figure 1), and moraine colonies were more likely to reject caterpillars than were creek colonies (Figure 2). None of the higher order interactions were significant.

The number of ants visiting a caterpillar increased significantly over time [Wilks' lambda $F_{(4,343)} = 32.39$, $P < 0.001$]. There were, however, significant time × diet [Wilks' lambda $F_{(4,343)} = 10.98$, $P < 0.001$] and time × site [Wilks' lambda $F_{(4,343)} = 6.20$, $P < 0.001$] interactions; the number of ants visiting a caterpillar increased significantly more over time for artificial-diet caterpillars than for leaf-diet caterpillars, and the number of ants visiting a caterpillar increased significantly more over time for creek colonies than for moraine colonies (Figure 3). For the between-subjects effects (which are hypotheses tests with the dependent variable equal to the total number of ants over the five time categories), there was a significant three-way interaction between diet, site, and instar [$F_{(1,346)} = 4.40$, $P = 0.037$], indicating that total

FIG. 2. The association between predator habitat type and predation response variables. "Moraine" refers to ant colonies located on a glacial moraine, while "creek" refers to colonies located adjacent to a creek below the moraine. The *y* axes represent percentages of all larvae ($N = 360$) offered to predators from specific habitats ($N = 6$ ant colonies) that received the predator response indicated on the *x* axis. For example, 40% of the caterpillars offered to ant colonies on the glacial moraine were never rejected. The predator responses on the *x* axis were categorical dependent variables in logit models.

ant visits (for the 5-min time period) depended on specific combinations of diet, colony, and instar. Late-instar, leaf-diet caterpillars offered to moraine colonies had the fewest ants visiting (7.0 ± 0.8), while late-instar, artificial-diet caterpillars offered to creek colonies had the most ants visiting (19.9 ± 0.9).

The total number of ants visiting caterpillars (for the 5-min time period) did not change between days [$F_{(4,340)} = 0.47$, $P = 0.760$]. There were significant diet [$F_{(1,340)} = 86.02$, $P < 0.001$], site [$F_{(1,340)} = 18.47$, $P < 0.001$], and instar [$F_{(1,340)} = 4.11$, $P = 0.044$] effects; total number of ants visiting was greatest for artificial diet caterpillars (16.9 ± 0.7), creek colonies (15.2 ± 0.7), and late instars (14.5 ± 0.6).

Experiment 2. A total of 60 caterpillars reared on artificial diets were offered to one creek colony and one moraine colony. The contingency table for rejections of artificial-diet caterpillars offered to colonies that received only artificial-diet caterpillars and colonies that received both artificial-diet and leaf-diet caterpillars revealed no significant association between colony type and level of rejection [$\chi^2_{(2)} = 4.009$, $P = 0.135$]. There was, however, a significant rela-

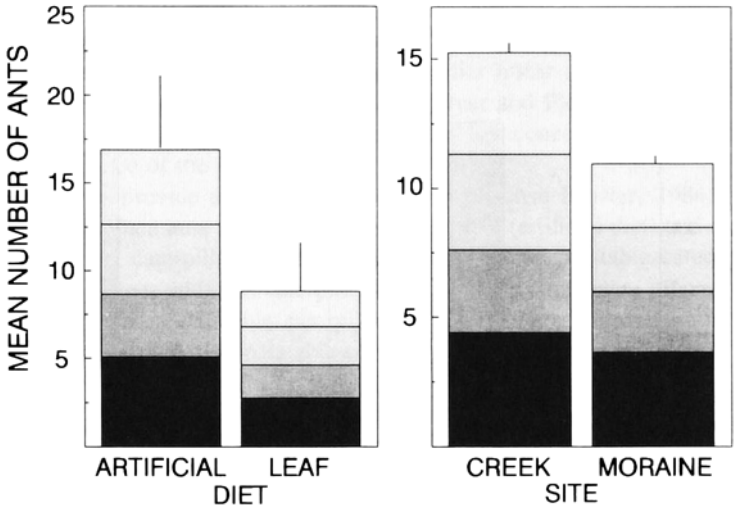

FIG. 3. The effects of larval diet and predator habitat site on ant recruitment. The *y* axes represent mean number of ants visiting caterpillars reared on specific diets and mean number of ants from specific sites visiting caterpillars. The number of ants for each minute is stacked upon the previous minute. The standard error bars are for the numbers of ants totaled over the 5 min.

tionship between colony type and level of escape [$\chi^2_{(1)} = 6.187$, $P = 0.013$). Artificial-diet caterpillars offered to colonies that received only artificial-diet caterpillars never escaped, while 9% of the artificial-diet caterpillars escaped at colonies that received caterpillars reared on both diets.

Experiment 3. We offered a total of 68 caterpillars to three ant colonies at the creek site. Percent aucubin was a significant predictor of escape [$\chi^2_{(1)} = 9.99$, $P = 0.002$] and rejection [$\chi^2_{(2)} = 14.84$, $P < 0.001$]. Likewise, percent catalpol was a significant predictor of escape [$\chi^2_{(1)} = 7.31$, $P = 0.007$] and rejection [$\chi^2_{(2)} = 14.66$, $P < 0.001$], indicating that iridoid glycoside concentration of larvae influenced predator response to these larvae. Caterpillars that escaped and were completely rejected had much higher iridoid concentrations than caterpillars that were eaten and had no rejections (Figure 4).

DISCUSSION

The results of the present study indicate that diet is an important component of the suite of defenses found in the specialist caterpillar, *J. coenia*. Larvae reared on leaves of *P. lanceolata*, which contain aucubin and catalpol (4–8%

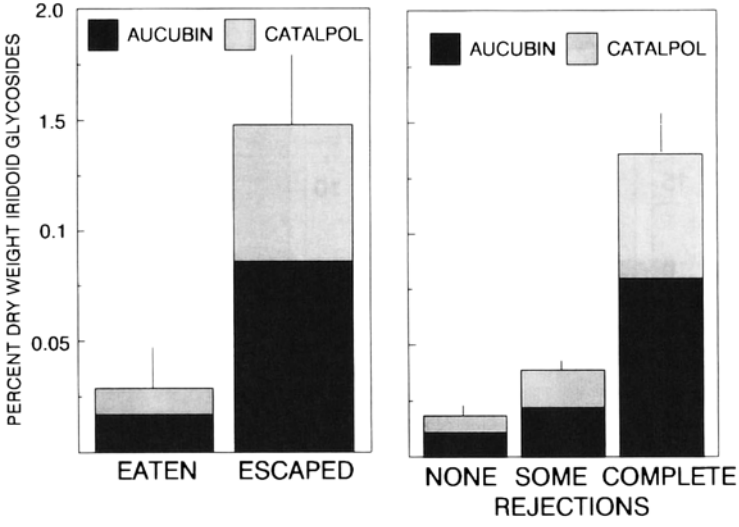

FIG. 4. The effects of iridoid glycoside concentrations (percent dry weight of aucubin and catalpol) on predation response variables. The *y* axes represent mean percent iridoids sequestered by caterpillars that received the predator response indicated on the *x* axis. For example, the mean percent dry weight of aucubin sequestered by caterpillars that escaped was 0.085%. The standard error bars are for total iridoids. The predator responses on the *x* axis were categorical dependent variables in logit models.

dry weight), are better protected against a voracious predator than larvae reared on an artificial diet with less than 0.4% dry weight iridoid glycosides. Sequestered iridoids seem to be the main component of the leaf-diet caterpillars' enhanced defenses. While it is true that iridoid concentrations are not the only differences between the two diets (i.e., one diet is plant material, the other is an agar-based gel), chemistry was the only obvious difference between the caterpillars reared on these different diets. For example, one might expect that the size and behavior of caterpillars would vary depending on their diet, but the caterpillars offered to the ants were matched for size and there were no apparent differences in their activity levels. Furthermore, the concentrations of iridoids found in caterpillars on both diets were good predictors of whether or not those caterpillars escaped predation or whether or not they were rejected by individual ants.

The fact that instar number was never a significant predictor of prey escape or predator rejection suggests that the low percentages of iridoid glycosides found in early instars are as effective as the higher percentages (especially for aucubin) found in later instars in deterring predation by ants. These chemicals,

therefore, seem to act as "qualitative" defenses against predators—high concentrations are not necessary for effective deterrence. Furthermore, certain invertebrate predators usually prefer smaller and earlier instar caterpillars as prey, including those that are chemically defended (Dyer and Floyd, 1993; also see Reavey 1993), so the protection of early instars by low concentrations of iridoids is further evidence of the efficacy of this defense.

Our results provide evidence of automimicry (sensu Brower, 1984) in *J. coenia* larvae. When ants were offered both palatable (artificial diet) and unpalatable (leaf diet) caterpillars of the same species, some palatable caterpillars were able to escape, while no caterpillars escaped when ants were offered only palatable caterpillars. Palatable caterpillars therefore were afforded some protection by the presence of unpalatable conspecifics. This is a rather weak example of automimicry, since most (>90%) of the palatable caterpillars were eaten by ants that had also been offered unpalatable caterpillars; additionally, there was no significant association between colony type and the level of rejection for palatable caterpillars. The difference in results between escape and rejection response variables may indicate that ants were *ignoring* or *avoiding* all caterpillars (regardless of diet) because they have received unpalatable buckeyes. Such avoidance would allow caterpillars to *escape*, as opposed to being examined and *rejected* by ants; as a result, the numbers of rejections for the palatable caterpillars offered to the same colonies would be lower (because they were simply ignored).

Despite the effectiveness of iridoids as a qualitative defense against *F. planipiles*, some leaf-diet caterpillars and caterpillars with high iridoids were still eaten by ants. In addition, *F. planipiles* showed no short-term ability to learn to avoid buckeye caterpillars, despite the fact that other species of predaceous ants are often able to learn prey avoidance quickly (Dejean, 1988; Bowers and Larin, 1989). The weak example of automimicry discussed above illustrates the ants' abilities to retain some sort of information within a 12-hr period, but there were no day-to-day changes in the colonies' responses to unpalatable caterpillars over a period of five days. This inability to retain information has the potential effect of minimizing selective pressures exerted by these and similar predators on distasteful prey, since there is not a consistent advantage provided by unpalatability. Aposematism, for example, is not likely to evolve in organisms exposed mostly to predators lacking the ability to learn or remember. Nevertheless, the presence of this chemical defense does provide a significant selective advantage over caterpillars that have not sequestered iridoids.

Ants from certain colonies were less likely to reject buckeyes. The creek colonies, which have high prey resources (mostly creek invertebrates; Dyer, *personal observations*), were more voracious: they recruited more, rejected fewer caterpillars, and the caterpillars were less likely to escape from these colonies. In contrast, the moraine colonies, which spend most of their time tending ho-

mopterans (Dyer, *personal observations*; McIver and Loomis, 1993) were much less interested in buckeyes as prey. This type of intraspecific variation is important to consider when conducting assays with predators or when examining the selective pressures exerted by specific predators. If our study had included only moraine colonies, a result such as iridoid sequestering caterpillars being no better defended than nonsequestering caterpillars might only reflect the fact that these particular colonies were not interested in preying on buckeyes. However, because there were no significant diet × colony interactions in the present study, the intraspecific variation in levels of predation by ants did not indicate that predators with fewer available prey were more likely than those with abundant prey to attack unpalatable prey, a pattern one might expect if prey abundance is an important selective pressure on prey choice.

In conclusion, we have demonstrated the defensive utility of a secondary compound sequestered by a specialist herbivore, and this contributes to a better understanding of how specialist herbivores protect themselves against invertebrate predators. We have also demonstrated intraspecific variation in prey preferences of an invertebrate predator, and such variation should be considered in the increasing numbers of studies on the natural enemies of insect herbivores.

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MASS-TRAPPING OF *Carpophilus* SPP. (COLEOPTERA: NITIDULIDAE) IN STONE FRUIT ORCHARDS USING SYNTHETIC AGGREGATION PHEROMONES AND A COATTRACTANT: DEVELOPMENT OF A STRATEGY FOR POPULATION SUPPRESSION

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Abstract—Experiments were conducted in southern New South Wales to evaluate the potential of mass-trapping using synthetic aggregation pheromones and a coattractant as a control option for *Carpophilus* spp. in stone fruit orchards. A cordon of 54 pipe and 54 funnel traps (one trap of each type per perimeter tree) baited with pheromones of *C. mutilatus* and *C. davidsoni* and coattractant (fermenting bread dough) was maintained around an apricot orchard for three weeks prior to harvest. The incidence of *Carpophilus* spp. in ripe fruit in the center of the orchard was significantly reduced compared to a nearby orchard or the perimeter trees containing traps. A cordon of 16 water-filled Magnet funnel traps baited with pheromones of *C. mutilatus* and *C. davidsoni* and coattractant was placed around a 9 × 9 block of trees in a peach orchard (single traps on alternate perimeter trees). This trapping regime significantly reduced infestation of fruit baits by *Carpophilus* spp. in the center tree over a period of six weeks compared to fruit baits in trap trees and distant (100 m) control trees. However, cordons of eight pheromone traps within 1 m of single trees or a single trap adjacent to a tree increased *Carpophilus* spp. infestation of fruit baits by up to 7.5 × compared to trees without pheromone traps. Mass-trapping based on perimeter positioning of pheromone

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traps (at a yet to be determined distance from protected trees) appears to show potential as a control strategy for *Carpophilus* spp. in stone fruit orchards during fruit ripening and harvest but traps too close to trees must be avoided. Development of a strategy for population suppression is discussed with respect to trap type, efficacy, positioning, and density; pheromone and coattractant delivery systems; and orchard sanitation.

Key Words—*Carpophilus mutilatus*, *Carpophilus davidsoni*, *Carpophilus hemipterus*, Coleoptera, Nitidulidae, aggregation pheromones, mass-trapping, stone fruit, population suppression.

INTRODUCTION

Three species of nitidulid beetles in the genus *Carpophilus* [*C. hemipterus* (L.), *C. mutilatus* Erichson, *C. davidsoni* Dobson] are major pests of ripening stone fruit in southern Australia (James et al., 1994, 1995). The recent identification and synthesis of aggregation pheromones for these species (Bartelt et al., 1990a, 1993; Bartelt and James, 1994) and demonstration of field activity (Bartelt et al., 1992, 1994a,b; James et al., 1994), has highlighted the potential of these semiochemicals for nitidulid management in stone fruit orchards.

Aggregation pheromones of *C. hemipterus*, *C. mutilatus*, and *C. davidsoni* are branched unsaturated hydrocarbons and are synergized by food volatiles such as those from fermenting bread dough or decomposing fruit (Bartelt et al., 1992; James et al., 1993). Wind-oriented pipe traps (Dowd et al., 1992) baited with rubber septa impregnated with 500 μg of synthetic pheromone and fermenting bread dough are highly effective in trapping large numbers of beetles. Bartelt et al. (1994b) reported trap catches of up to 63,500 *C. mutilatus* per trap per week in a California date garden and up to 20,179 *C. davidsoni* were trapped in a single trap in one week in a peach orchard in southern Australia (James, unpublished observation). Even larger numbers of beetles are caught in traps using higher doses of pheromone. Pheromone (5000 μg) generally increases trap catches of *C. hemipterus* and *C. mutilatus* by 5–8 × compared to traps using 500 μg of pheromone (Bartelt et al., 1994a; James et al., 1994). Finally, James et al. (1996) showed that pheromone-baited, water-based funnel traps were about five times more effective than wind-oriented traps in capturing *Carpophilus* spp. Clearly, synthetic pheromones in combination with a host-related coattractant have the potential to be an important weapon for managing *Carpophilus* spp. populations in stone fruit orchards. They could be used as the basis of a sensitive monitoring system (to better time chemical sprays) or for mass-trapping beetles to suppress orchard populations below damaging levels. Mass-trapping as a control option for *Carpophilus* spp. was first suggested by Warner (1960, 1961), who reported a reduction in infestation levels in figs through the use of traps containing fermenting figs as a bait.

Here, we report the results from a series of field experiments aimed at identifying and evaluating a strategy using synthetic pheromones for suppression of *Carpophilus* spp. populations in stone fruit orchards.

METHODS AND MATERIALS

Synthetic Pheromones

Pheromones for *C. hemipterus*, *C. mutilatus*, and *C. davidsoni* were used in these experiments. The pheromone for *C. hemipterus* consisted of a 100:31:11:8 blend of (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene, (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene, (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-decatetraene, and (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-undecatetraene, respectively (Bartelt et al., 1992). The pheromone for *C. mutilatus* consisted of a 100:7 blend of (3*E*,5*E*,7*E*)-5-ethyl-7-methyl-3,5,7-undecatriene and (3*E*,5*E*,7*E*)-6-ethyl-4-methyl-3,5,7-decatriene (Bartelt et al., 1993). The pheromone for *C. davidsoni* consisted of a 100:9:31:160 blend of (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene, (3*E*,5*E*,7*E*)-6-ethyl-4-methyl-3,5,7-decatriene, (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene, and (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-undecatetraene (Bartelt and James, 1994). The synthetic pheromones were purified only by distillation and open column chromatography on silica gel. These procedures did not remove the small amounts of *Z* isomers produced in the syntheses (Bartelt et al., 1990b), but there is no evidence that these (presently unavoidable) impurities are detrimental to pheromonal activity (Bartelt et al., 1992). Pheromones were appropriately diluted with hexane and stored in a freezer until needed. Concentrations of components were determined by gas chromatography on diluted aliquots (instrumentation as described by Bartelt et al., 1990a). Pheromone solutions were applied to rubber septa (11 × 20 mm, red rubber, Aldrich Chemical Co., Milwaukee, Wisconsin) followed by 300 μl of methylene chloride. Once the liquid had soaked into the septa, they were aired in a fume hood for 1-hr and stored in a freezer in tightly closed bottles until needed.

Septa used in suppression traps were prepared with 5000 μg each of *C. mutilatus* and *C. davidsoni* pheromone per septum. Prior monitoring indicated *C. hemipterus* was uncommon, therefore, it was not included in the suppression trials. Septa used in monitoring traps were loaded with 5000 μg each of pheromones for the three species.

Traps and Coattractant Baits

Suppression experiments were conducted using either the wind-oriented pipe trap of Dowd et al. (1992), a simple funnel trap (Faulder funnel) designed as part of this research (James et al., 1996), or the commercially available

Magnet funnel trap (Agrisense, Pontypridd, Glamorgan, UK). The pipe trap has been used in most of the pheromone studies previously conducted by the authors (e.g., Bartelt et al., 1994a; James et al., 1994). Traps were constructed from white PVC piping and assembled in the form of a T. Beetles entered the trap through a cone-shaped piece of screen in one horizontal branch and ended up in a plastic 140-ml gauze-bottomed plastic bottle attached to the bottom of the trunk pipe. The trap design incorporates a fin that enables orientation with the wind so that the trap entrance is always accessible to beetles approaching the trap from downwind. No killing agent was used, and screen barriers prevented beetles from contacting the bait, which is in the other horizontal branch.

The Faulder funnel trap was constructed from a 12-cm length (11 cm diameter) of white PVC piping with red plastic funnels fitted at either end; the top funnel had its base inside the pipe and that of the lower funnel was directed downward. The base of the top funnel (4 cm diameter) was covered with nitidulid-proof gauze except for a central opening. The bottom funnel screwed onto the pipe via a flange and led into a 140-ml solid-bottomed collecting bottle half-filled with water. The Magnet funnel trap is a simple funnel design primarily used for trapping moths. James et al. (1996) showed this trap, when used with water to a depth of 2–4 cm, to be highly effective for *Carpophilus* spp., trapping 3–7× as many beetles as dry pipe or funnel traps.

Fermenting whole wheat bread dough was used as the pheromone synergist (coattractant) (approximately 10 ml/trap) held in a 20-ml glass tube with a gauze lid. The coattractant and pheromone septum were placed in the bait compartment of the pipe trap, taped to the inside of the Faulder funnel pipe or taped to the inside of the downward projecting funnel of the Magnet™ funnel trap. In the orchard-scale suppression experiment, all traps were suspended from tree branches at about 1.5 m above the ground. Traps were serviced weekly, beetles collected, and dough replaced. Pheromone septa were replaced fortnightly. *Carpophilus* spp. collected in suppression traps were counted in the laboratory, while those trapped in population-monitoring traps and collected from fruit were also identified to species using Dobson (1954, 1964).

Experimental Design and Sites

An orchard-scale suppression experiment was conducted in a commercial orchard followed by a series of smaller, within-orchard experiments at another site aimed at suppressing *Carpophilus* spp. populations on single trees. Both sites were located near Leeton in the Murrumbidgee Irrigation Area of southern New South Wales.

Suppression of Carpophilus *spp. in a Commercial Apricot Orchard*

This experiment was conducted in a 1-ha apricot (cv Trevatt) orchard containing 187 trees (10 × 20 rows) with 5-m spacing. Three pipe traps were

placed in the center of the block (10 m apart and 30 m from perimeter) in early spring (September 28, 1994) to monitor *Carpophilus* spp. activity Suppression traps (one pipe trap and one Faulder funnel trap) were placed in each perimeter tree ($N = 54$) on December 7, 1994, when *Carpophilus* spp. populations began to increase dramatically. Traps were hung on opposite outside edges of canopies and were aimed at intercepting incoming beetles in addition to luring beetles already within the orchard. Harvesting commenced on December 27, and trapping terminated on January 4. The orchard was bordered on two sides by citrus but no ripe fruit or obvious *Carpophilus* spp. population was present. Three methods were used to assess *Carpophilus* spp. populations inside and outside the orchard.

Fruit Examination. Damaged (split or bird-pecked) ripe hanging fruit and fallen fruit were collected on December 29 and examined individually for *Carpophilus* spp. Samples were collected randomly, either from the center (i.e., >20 m from perimeter trees) of the experiment block (fallen fruit: $N = 30$) or taken from central or perimeter trees ($N = 40$ ripe fruit/area). Ripe and fallen fruit were also sampled from the center of an apricot orchard 1 km from the experiment orchard.

Fruit Baits. Three trays of ripe, punctured apricots (20/tray) were placed randomly in the center of the block (1 m above the ground in tree crotches) on December 23 and 30. Trays were retrieved after five days and examined in the laboratory for beetles. On December 23 this procedure was replicated in the nearby orchard. On December 30 three fruit bait trays were placed in citrus trees adjacent (15 m) to the experiment orchard.

Pipe (monitoring) Traps. Beetle catches from the center of the block were collected prior to and during the experiment.

Suppression of Carpophilus *spp. in Single Peach Trees*

Four separate experiments were conducted using pheromone and fermenting bread dough-baited Magnet funnel traps to protect provisioned ripe fruit in single peach trees from *Carpophilus* spp. All experiments were conducted consecutively during January–March 1995 in an unsprayed orchard with no naturally occurring ripe fruit.

Experiment A. The aim of this experiment was to protect provisioned, overripe fruit from *Carpophilus* spp. by placing a cordon of pheromone traps around the tree. Two pairs of trees forming the four corners of a square were selected in the center of the block. Two trees were "treated" with pheromones (diagonally opposite) and the other two were untreated controls. Trees were separated from each other by at least 10 m. Eight steel posts were erected around each of the treatment trees with an interpost distance of 1.5 m. A Magnet funnel trap filled with water to a depth of 3–4 cm was attached to the top of each post at a height of about 1.5 m. The distance from traps to tree canopy was approx-

imately 1 m. All trees were baited with three plastic containers (1 liter) with drainage holes, each containing four overripe, punctured apricots. The containers were tied to branches and well separated within the tree canopy. After five days beetles were removed from the traps and fruit baits and counted in the laboratory. Beetles in the fruit baits were also identified to species.

Experiment B. In this experiment, the effect of trap height on protecting fruit from beetle attack was investigated. A cordon of eight traps was positioned at two heights: 2.5 m above ground level (approximately at upper canopy height) and at ground level. Two overripe peaches, each with a single puncture, were used in each fruit bait and the experiment ran for three days. An additional two distant (100 m) control trees were used in this and the following two experiments.

Experiment C. In this experiment one trap on a single post was used to protect fruit baits on trees, rather than a trap cordon. Overripe apricot fruits with a single puncture were used in the baits and the experiment ran for three days.

Experiment D. The strategy in this experiment was to protect fruit baits from *Carpophilus* spp. on a single tree using a cordon of pheromone traps positioned 20–30 m away. A 9 × 9 block (50 × 40 m) of trees within the orchard was selected and traps ($N = 16$) placed in alternate trees around the perimeter of this block. The protected tree was in the exact center of the block. Three fruit baits (each containing two overripe, single punctured peaches) were placed in the protected tree and each of the four corner trap trees. Baits were also placed in two tree (controls) 100 m either side of the cordoned block. The experiment ran for six weeks with weekly servicing of traps (replacement of dough and pheromone), collection of beetles from traps and fruit baits, and replacement of fruit in bait containers.

Two pipe traps were also placed in this orchard away from the experiment area throughout the single tree experiments, to monitor *Carpophilus* spp. incidence.

Data were transformed to the log $(x + 1)$ scale or arcsin-transformed (proportions) and subjected to analysis of variance and least significant difference procedures.

RESULTS

Suppression of Carpophilus *spp. in a Commercial Orchard*

A total of 22,669 beetles was caught in the suppression traps during this experiment, with significantly greater numbers captured in the Faulder funnel traps ($P < 0.05$) (Table 1). The number of beetles trapped in the central monitoring traps fell significantly after the suppression traps were installed. However, trappings did not decline further during the experiment and were comparable

TABLE 1. *Carpophilus* SPP. BEETLES TRAPPED IN SUPPRESSION AND MONITORING TRAPS IN A COMMERCIAL APRICOT ORCHARD, DECEMBER 7, 1994–JANUARY 4, 1995[a]

	Suppression traps[b]					Monitoring traps (pipe)[c]			
	Pipe(p)		Funnel(f)				Species (%)		
Date	Total	Mean ± SE	Total	Mean ± SE	Total p + f	Mean[d] ± SE	*davidsoni*	*mutilatus*	*hemipterus*
ovember 30				Presuppression		62 ± 5b	79	10	11
ecember 7						77 ± 10b	75	20	5
14	880	16 ± 2	6757	125 ± 14*	7637	23 ± 5a	81	15	4
21	1647	31 ± 4	3261	60 ± 5*	4908	28 ± 5a	85	10	5
29	1442	27 ± 4	3043	57 ± 6*	4485	24 ± 8a	94	2	4
nuary 4	2281	42 ± 4	3358	62 ± 4*	5639	30 ± 4a	93	5	2
ntire riod	6250	29 ± 5	16419	76 ± 14*	22669	26 ± 1	85	10	5

/alues followed by an asterisk are significantly greater than corresponding value for suppression pipe traps $P < 0.05$).

Suppression traps (54 pipe, 54 funnel) were placed in perimeter trees and baited with 5000 μg each of *C. davidsoni* and *C. mutilatus* pheromones and fermenting bread dough.

Monitoring traps (three pipe) were placed in the center of the orchard and baited with 500 μg each of *C. davidsoni*, *C. mutilatus*, and *C. hemipterus* pheromones and fermenting bread dough.

Values followed by same letter not significantly different ($P > 0.05$).

to those recorded in the suppression pipe traps (n.b., monitoring traps contained pheromones for *C. davidsoni*, *C. mutilatus*, and *C. hemipterus*).

Damaged, ripe fruit sampled from the center of the orchard on December 29 had a very low level of beetle infestation. In contrast, virtually all fruit sampled from perimeter trees (containing suppression traps), contained beetles (Table 2). This was also the case for ripe, damaged fruit sampled from the nearby orchard. *C. davidsoni* was the only species present in ripe fruit. There was little difference in overall numbers of beetles in fallen fruit or fruit bait samples taken from the experiment orchard, the nearby orchard or the adjacent citrus block (Tables 2 and 3). However, the ratio of *C. davidsoni* plus *C. mutilatus* (the species targeted by the suppression traps) to *C. hemipterus* in baits and fallen fruit was reduced within the experiment orchard (22–50%) compared to adjacent citrus (69%) and the nearby orchard (62–81%) (Tables 2 and 3). *C. davidsoni* and *C. mutilatus* also dominated captures in the monitoring traps (89–98%) (Table 1).

TABLE 2. INFESTATION AND SPECIES INCIDENCE OF ADULT *Carpophilus* SPP. COLLECTED FROM SAMPLES OF RIPE AND FALLEN FRUIT AT HARVEST IN A COMMERCIAL APRICOT ORCHARD WITH PERIMETER SUPPRESSION TRAPS AND A NEARBY ORCHARD WITH NO SUPPRESSION TRAPS

Ripe fruit (split or bird-damaged)[a]					
Orchard with suppression traps[b]				Nearby orchard center trees[c]	
Center trees		Perimeter (trap) trees			
Fruit infested (%)	Beetles/fruit (mean ± SE)	Fruit infested (%)	Beetles/fruit (mean ± SE)	Fruit infested (%)	Beetles/fruit (mean ± SE)
5	0.1 ± 0.1a[d]	90	4.8 ± 0.9b	85	5.2 ± 0.4b

Fallen fruit							
Orchard with suppression traps[b]				Nearby orchard[c]			
Beetles/fruit (mean ± SE)	Species % (mean ± SE)			Beetles/fruit (mean ± SE)	Species % (mean ± SE)		
	C. hemipterus	*C. davidsoni*	*C. mutilatus*		*C. hemipterus*	*C. davidsoni*	*C. mutilatus*
2.0 ± 0.2a	78 ± 1[e]	22 ± 1	0	1.8 ± 0.1a	38 ± 4	62 ± 4[e]	0

[a] All beetles in ripe fruit were *C. davidsoni*.
[b] Suppression traps (54 pipe, 54 funnel) were placed in perimeter trees and baited with 5000 μg each of *C. davidsoni* and *C. mutilatus* pheromones and fermenting bread dough.
[c] 1 km from orchard with suppression traps.
[d] Numbers in rows followed by the same letter are not significantly different ($P > 0.05$).
[e] Significantly greater than for same species in the other location ($P < 0.05$).

TABLE 3. INFESTATION AND SPECIES INCIDENCE OF ADULT *Carpophilus* SPP. COLLECTED FROM FRUIT BAITS AFTER FIVE DAYS IN COMMERCIAL APRICOT ORCHARD WITH PERIMETER SUPPRESSION TRAPS, FRUIT BAITS IN NEARBY ORCHARD WITH NO TRAPS, AND CITRUS GROVE ADJACENT TO ORCHARD WITH TRAPS

Date baits provided	Orchard with suppression traps[a]				Nearby orchard[b]				Adjacent citrus grove			
	Beetles/fruit (mean ± SE)	Species % (mean ± SE)			Beetles/fruit (mean ± SE)	Species % (mean ± SE)			Beetles/fruit (mean ± SE)	Species % (mean ± SE)		
		C. hemipterus	*C. davidsoni*	*C. mutilatus*		*C. hemipterus*	*C. davidsoni*	*C. mutilatus*		*C. hemipterus*	*C. davidsoni*	*C. mutilatus*
Dec. 23	11.7 ± 2.6a[c]	50 ± 7[d]	48 ± 6	2 ± 1	11.4 ± 0.8a	19 ± 4	70 ± 8[d]	1 ± 5[d]				
Dec. 30	5.1 ± 0.7a	59 ± 4[d]	36 ± 6	5 ± 2					6.4 ± 2.5a	31 ± 4	56 ± 4[d]	13 ± 2[d]

[a] Suppression traps (54 pipe, 54 funnel) were placed in perimeter trees and baited with 5000 μg each of *C. davidsoni* and *C. mutilatus* pheromones and fermenting bread dough.
[b] 1 km from orchard with suppression traps.
[c] Numbers in rows followed by the same letter are not significantly different ($P > 0.05$).
[d] Significantly greater than for the same species in the other location ($P < 0.05$).

Suppression of Carpophilus *spp. Populations in Single Trees*

Experiment A. The eight trap cordons surrounding the two pheromone-protected trees caught 1767 and 1445 beetles, respectively. Significantly greater numbers of beetles were found in the trap tree fruit baits than in the untreated tree baits ($P < 0.05$) (Table 4).

Experiment B. Trap cordons at a height of 2.5 m around each tree captured totals of 892 and 758 beetles, respectively. Fruit baits in these trees collected a mean of 45 beetles/bait which was 3.5× greater than the nearby untreated trees and 7.5× greater than the distant untreated trees (Table 4). Trap cordons set at ground level around each tree captured 536 and 452 beetles, respectively. Fruit baits in these trees collected a mean of 87 beetles/bait, which was similar to the levels in the nearby untreated trees but almost four times the number of beetles in distant untreated trees (Table 4).

Experiment C. The single traps adjacent to each tree captured 20 and 30 beetles, but fruit baits in these trees contained a mean of 45 beetles/bait, significantly greater than beetle numbers in control tree baits (Table 4).

Experiment D. The 16-trap cordon significantly suppressed beetle numbers in fruit baits on the center tree in all weeks compared to numbers in fruit baits on trap and control trees ($P < 0.05$) (Table 5). The species targeted by the suppression traps (*C. davidsoni* and *C. mutilatus*) comprised the majority of beetles found in fruit baits on the center and trap trees in all weeks. *C. hemipterus* was significantly more abundant than *C. davidsoni*/*C. mutilatus* in con-

TABLE 4. INFESTATION OF ADULT *Carpophilus* SPP. IN FRUIT BAITS IN SINGLE PEACH TREES CORDONED BY EIGHT MAGNET FUNNEL TRAPS COMPARED TO UNCORDONED TREES[a]

Experiment	Trap height[b]	Duration (days)	Beetles/trap (mean ± SE)	Beetles/fruit bait in trees (mean ± SE)		
				Pheromone treated	Nearby untreated[c]	Distant untreated[d]
A	1.5 m	5	200 ± 25	124 ± 40a	37 ± 6b	—
B	2.5 m	3	103 ± 10	45 ± 6a	13 ± 3b	6 ± 1c
	ground level	3	62 ± 5	87 ± 20a	78 ± 10a	23 ± 6c
C	1.5 m	3	25 ± 3	37 ± 8a	14 ± 5b	1 ± 0.5c

[a] In experiment C cordons were replaced with a single trap. Values in rows followed by different letters are significantly different ($P < 0.005$).

[b] Traps approximately 1 m from tree canopy and baited with 5000 μg each of *C. davidsoni* and *C. mutilatus* pheromones and fermenting bread dough.

[c] 10 m from pheromone-treated trees.

[d] 100 m from pheromone-treated trees.

TABLE 5. INCIDENCE OF ADULT *Carpophilus* SPP. OVER SIX WEEKS IN FRUIT BAITS IN SINGLE TREE IN CENTER OF SMALL PEACH TREE BLOCK (50 m × 40 m) CORDONED BY 16 MAGNET FUNNEL SUPPRESSION TRAPS BAITED WITH AGGREGATION PHEROMONE (5000 μg EACH OF *C. davidsoni* AND *C. mutilatus* PHEROMONES) AND FERMENTING BREAD DOUGH, FRUIT BAITS IN CORNER TRAP TREES, AND TWO CONTROL TREES (100 m FROM CORDONED BLOCK)[a]

Week	Beetles/ suppression trap (mean ± SE)	Beetles/fruit bait (mean ± SE)								
		Center tree			Corner trap trees			Control trees		
		C. mutilatus/ *C. davidsoni*	*C. hemipterus*	All spp.	*C. mutilatus*/ *C. davidsoni*	*C. hemipterus*	All spp.	*C. mutilatus*/ *C. davidsoni*	*C. hemipterus*	All spp.
1	230 ± 27	23 ± 4a	1 ± 2b	24 ± 4a	51 ± 8c	12 ± 5d	63 ± 11c	18 ± 5a	48 ± 16c	66 ± 22c
2	144 ± 16	16 ± 1a	1 ± 1b	17 ± 1a	43 ± 18c	0	43 ± 18c	19 ± 9a	10 ± 4d	29 ± 12d
3	46 ± 6	9 ± 5a	0	9 ± 5a	26 ± 4b	5 ± 2a	31 ± 6b	11 ± 3a	24 ± 5b	35 ± 7b
4	105 ± 9	8 ± 4a	0	8 ± 4a	32 ± 2b	5 ± 2a	37 ± 3b	25 ± 4d	2 ± 1c	27 ± 5d
5	54 ± 4	1 ± 1a	0	1 ± 1a	10 ± 2b	2 ± 2a	12 ± 3b	6 ± 2c	15 ± 5bd	21 ± 7d
6	23 ± 3	0	0	0	4 ± 2a	0	4 ± 2a	0	1 ± 0	1 ± 0
Whole period	100 ± 29	9.5 ± 3a	0.3 ± 0.2b	9.8 ± 4a	27.7 ± 7c	4 ± 2d	31.7 ± 8c	13.2 ± 3a	16.7 ± 7a	29.8 ± 8c

[a]Values in rows followed by a different letter are significantly different ($P < 0.05$).

trol tree fruit baits in three of the six weeks but overall there was no significant difference in their relative abundance ($P > 0.05$).

The *Carpophilus* population in this orchard, as determined by the monitoring traps, was dominated by *C. davidsoni* and *C. mutilatus* with these species accounting for 60% and 26%, respectively, of beetles trapped during the single tree experiments.

DISCUSSION

Synthetic aggregation pheromones of *Carpophilus* spp. have enormous potential for monitoring the populations of these pests. In Australian stone fruit production, the use of pheromones prior to harvest should allow growers to monitor the presence and population build up of *Carpophilus* spp., enabling precise judgement for the need and optimal timing of insecticide treatments. The potential for using synthetic pheromones for control of *Carpophilus* has remained largely unexplored; this study is the first to investigate mass-trapping based on pheromones as a control option for *Carpophilus* spp. The data obtained from this series of experiments suggest that the prospects are good for development of pheromone-based mass-trapping as a management strategy for orchard suppression of *Carpophilus* spp. By testing various ways of using pheromones and/or traps to mass-trap beetles and protect fruit, we have highlighted strengths and weaknesses that can be targeted in future research.

Pheromone trapping studies over a number of seasons have shown *Carpophilus* spp. in all major stone fruit growing regions of southern Australia are most abundant during spring. These populations decline naturally (probably due to shortage of breeding sites, e.g., ripe fruit) to a fairly low level in late spring/early summer (late November–early December) (James et al., 1993, 1994, 1995). Mass-trapping as a control strategy is therefore most appropriately timed to begin after the natural decline has occurred and before populations begin increasing again, fueled by the increasing seasonal availability of ripening fruit. Mass-trapping of the large spring populations of *C. mutilatus* that can occur in California date gardens, as suggested by Bartelt et al. (1994b), may be appropriate because of the continuous availability of host resources, which prevents a marked population decline.

In the commercial orchard experiment mass-trapping in perimeter trees for three weeks before harvest appeared to have some impact on *Carpophilus* spp. populations. Numbers of beetles caught in the center of the orchard fell after mass-trapping started and damaged, ripe fruit in the center of the orchard at harvest were largely beetle-free. This contrasted with fruit in perimeter (trap) trees and fruit in the nearby orchard, which were heavily infested with beetles. In experiment D beetle populations in fruit baits on a central tree in a small

block cordoned by 16 funnel traps were also suppressed in comparison to populations in fruit baits on the trap trees and distant control trees. While mass-trapping in a perimeter zone appears to reduce beetle populations within a block, large populations occur near the traps. In experiments A–C beetle numbers in fruit baits on trees cordoned by eight traps were up to 7.5× greater than those on trees not surrounded by pheromone traps. Height of the traps did not appear to influence this response. Clearly, not all beetles attracted to pheromones emanating from traps are actually trapped. Once drawn to the vicinity of a pheromone trap they can be diverted to nearby ripe fruit. The positioning of pheromone traps is therefore an important consideration for both mass-trapping and monitoring *Carpophilus* spp. Further studies are needed to determine the extent of the potential damage zone around pheromone traps. Stone fruit orchards in the MIA are often bordered by citrus or grapevines and suppression traps could be positioned in the perimeter zones of these crops, which are not affected by *Carpophilus* spp.

Although very small numbers of beetles were observed in ripe fruit in the center of the commercial orchard compared to the perimeter and the nearby orchard, this was not the case in samples of fallen fruit and the fruit baits. Total numbers of beetles in fallen fruit or fruit baits were comparable in the experiment orchard, the nearby orchard, and adjacent citrus. However, *C. hemipterus* dominated beetle populations in fallen fruit and fruit baits in the experiment orchard, but *C. davidsoni* dominated elsewhere. This suggests that *C. davidsoni* was suppressed within the orchard allowing the nontargeted *C. hemipterus* (only pheromones of *C. davidsoni* and *C. mutilatus* were used in suppression traps) to infest fruit at a higher rate. *C. hemipterus* attracted to the orchard by ripening fruit were not intercepted by the suppression traps, as the other two species were, and therefore became the dominant species within the orchard. Although some cross-attraction does occur with *Carpophilus* spp. pheromones, the pheromones of *C. davidsoni* and *C. mutilatus* are poor attractants for *C. hemipterus* (Bartelt et al., 1994b; Bartelt and James, 1994). Season-long pheromone trapping of *Carpophilus* spp. (using pheromones of all three species) in three other stone fruit orchards in the MIA during 1994/95 confirmed *C. davidsoni* was the dominant species (total captures of *C. davidsoni* 72%, *C. mutilatus* 26%, *C. hemipterus* 2%) (James unpublished data). Similar results, contrary to those obtained from fruit samples were obtained from the monitoring traps in the center of the orchard. Pipe traps baited with pheromone and dough attract *Carpophilus* spp. from distances of up to 350 m (James, unpublished data). Thus, beetles caught in the orchard monitoring traps may have reflected the *Carpophilus* spp. population over a wider area than just the pheromone-treated orchard. Significant differences in species ratio also occurred in fruit baits from pheromone-treated and untreated trees in the single tree experiments in 1995.

In the small block experiment (D), beetle numbers in fruit baits on the

protected tree were always very small but invariably dominated by *C. davidsoni* and/or *C. mutilatus*. These were individuals that presumably had leaked through the suppression trap cordon (baited with *C. davidsoni* and *C. mutilatus* pheromones). The large numbers of beetles in fruit baits on the trap trees were dominated by *C. davidsoni* and *C. mutilatus*, but on the distant control trees *C. hemipterus* was sometimes dominant. *C. hemipterus* presumably did not invade the protected block as it did in the commercial orchard because the ripening fruit stimulus was insufficient (the only ripe fruit within the protected block were in the three fruit baits on the central tree). The alteration of species ratio in the small block experiment was indicative of suppression traps drawing the *C. davidsoni* and *C. mutilatus* populations away from the rest of the block, leaving *C. hemipterus* as the dominant species.

A number of parameters were used in these experiments to determine the effect of mass trapping on *Carpophilus* spp. populations (total counts and relative incidence of *Carpophilus* spp. in suppression traps, monitoring traps, and samples of ripe and fallen fruit and fruit baits). As discussed above, the long-range attraction of pheromone-baited monitoring traps prevents these from giving an accurate indication of *Carpophilus* spp. numbers in the immediate trap vicinity. The use of coattractant only (fermenting bread dough) in these traps, reducing their long-distance attraction, should give better results. Beetle damage to hanging ripe fruit is likely to be the best criterion for judging the suppressive effect of mass trapping on *Carpophilus* spp. populations, as this defines their economic importance. Observations indicate that small populations of *Carpophilus* spp. can occur in stone fruit orchards (utilizing fallen fruit as hosts) without causing economic damage to fruit on the trees (James, unpublished observations).

Success in suppressing *Carpophilus* spp. populations will depend on the efficacy of the traps used. James et al. (1996) showed water-based funnel traps to be the most effective in capturing large numbers of beetles. The number of traps necessary for suppressing beetle populations in a given area must also be determined. Data from current studies on the range and efficiency of *Carpophilus* spp. pheromone traps should indicate optimal trap spacing and density. Other aspects that require further research include development of improved delivery systems for the pheromones and the coattractant. Pheromone-impregnated rubber septa only work at maximum efficiency for about a week (Bartelt et al. 1994a) and full activity over a longer period is clearly desirable. A longer lasting and possibly more effective synthetic coattractant would also improve the practical aspects of mass trapping. It is possible that a lower quantity of pheromone (500 μg instead of 5000 μg) might improve trapping efficiency, increasing the proportion of beetles entering maps and minimizing the numbers of untrapped beetles attracted to the trap vicinity.

Pheromone-based mass trapping is unlikely to totally remove *Carpophilus* spp. populations from orchards. However, in combination with other measures,

such as the removal of fallen fruit and limiting the impact of nearby *Carpophilus* spp. populations through sanitation or spraying, mass trapping has the potential to assist in effective integrated control of these increasing pests of Australian stone fruit.

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AGGREGATION OF THE SCARAB BEETLE *Holotrichia consanguinea* IN RESPONSE TO FEMALE-RELEASED PHEROMONE SUGGESTS SECONDARY FUNCTION HYPOTHESIS FOR SEMIOCHEMICAL

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Abstract—The pheromone system of the scarab beetle *H. consanguinea*, an agricultural pest native to India, was investigated by extracting abdominal glands of females with dichloromethane and ether and analyzing them by GC-MS. Indoor bioassays with the natural product separated on a silica gel column showed that males responded to the hexane–ether (80:20) fraction by displaying a clear sexual behavior. Although the indoor bioassay-oriented approach did not lead to the identification of the active compound(s), field tests of candidate chemicals—anisole, indole, and phenol—showed that beetles responded strongly to anisole. Males and females were captured in anisole-baited traps in a ratio close to 1:1. Nearly the same sex ratio was also observed in beetles captured over the time during the flight activity of the beetles in the field. Because no clear evolutionary basis exists for why competing females are attracted to the semiochemical, it was simply referred to as a "female-released pheromone."

Key Words—Coleoptera, Scarabaeidae, anisole, indole, phenol, aggregation behavior.

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INTRODUCTION

Although we cannot rule out the existence of signals that arise *de novo*, perhaps most insect signals are postulated to have arisen from characteristics originally having other functions (Otte, 1974). For the evolution of chemical signals, either the compound or the sensory apparatus to detect these compounds may have preceded the evolution of functional communication. Thus, pheromones may have originated from hormones, plant allelochemicals, cuticular constituents, the insect's own defensive compounds, metabolic waste products, or some combination of these or other factors. The original functions of the chemicals may or may not be eventually lost. In the origin of chemical communication, there are several examples that seem to fit this secondary function hypothesis (reviewed in Haynes and Potter, 1995).

Recent studies on the chemical ecology of scarab beetles have led to the identification of sex pheromones of various species in the subfamilies Melolonthinae and Rutelinae (Leal, 1996). Interestingly, most of the chemicals utilized by the more advanced group (Rutelinae) are likely to be fatty acid derivatives, whereas the more primitive group (Melolonthinae) utilizes ubiquitous compounds per se or as an immediate precursor. Based on the facts that the sex pheromones of melolonthines are largely produced in huge amounts (Leal et al., 1993), enantiomeric purity is not critical (Matsuyama et al., 1994), and since most of them have antimicrobial activity (Leal and Kurata, 1994), it was suggested by Leal (1996) that these semiochemicals may have evolved from a primary defensive role.

Some melolonthine species, notably *Holotrichia* and *Phyllophaga* species, emerge at dusk and aggregate in a small time window. Females of *H. parallela*, for example, display a calling behavior only for ca. 15 min (Leal et al., 1993). It is conceivable that this behavior could also have advantages other than mate finding, such as minimizing the effect of a predator. In such cases, selection could favor nonspecific pheromones that would attract both sexes equally. We now report that a female-released pheromone of *H. consanguinea*, an economically important pest in India, is responsible for the aggregation of male and female beetles.

METHODS AND MATERIALS

Analytical Procedures. Gas chromatography (GC) was carried out on a Hewlett-Packard 5890 II Plus instrument equipped with a split/splitless injector, electronic pressure control, a flame ionization detector, and an HP 3365 Series II Chemstation. High-resolution GC analyses were performed with polar and nonpolar capillary columns: HP-Innowax and HP-5MS (30 m × 0.25 mm ×

0.25 μm; Hewlett-Packard), respectively. These columns were operated at 50°C for 1 min, increased to 180°C at a rate of 4°C/min, held at this temperature for 1 min, increased again to 230°C at a rate of 10°C/min, and finally held at this temperature for 20 min. Low-resolution mass spectrometry (MS) was carried out either with an HP 5890 II Plus gas chromatograph linked to a mass selective detector MSD 5972 (Hewlett-Packard) or on an Hewlett-Packard gas chromatograph electron ionization detector GCD series. Chromatographic resolution was achieved on HP-5MS and HP-Innowax columns operated under the same conditions for GC.

Extracts. A kit containing all the materials necessary for the extractions of pheromone glands was shipped to India, where the pheromone was collected. Specially prepared solvents were used, a small portion being kept as a blank. Abdominal tips of calling females were excised with forceps, cleaned, and then washed with either dichloromethane or ether. Extracts of 10–20 females were transferred to ampules, flame-sealed, and shipped to Japan.

Isolation of Pheromone. In Japan, the extracts were analyzed by GC and GC-MS. An extract of 80 females equivalents (FE) was fractionated on a silica gel column (Wako C-200) by successively eluting with hexane–ether mixtures in the following order: 100:0, 95:5, 90:10, 80:20, 50:50, and 0:100. All the fractions were concentrated to 0.5 FE, and two thirds of each fraction was sent back to India for bioassay, whereas the remainder was used for chemical analysis.

Indoor Bioassay. The arena consisted of 75 × 60 × 60-cm wire gauge cages, containing a layer of moist soil (8 cm thick) on the bottom. Males collected in the field, when they were about to copulate, were released in each cage (45 per cage) one day before the bioassay. Fractions were transferred from the ampule to a filter paper that was placed inside the arena. Behavioral responses were observed from 19:00 to 21:30 hr.

Field Experiments. Evaluation of the candidate pheromones was conducted at the fields of the Agricultural Research Station, Durgapura-Jaipur, Rajasthan in the summers of 1994 and 1995. Funnel traps (Japan Tobacco, Inc., Tokyo), were baited with synthetic lures incorporated into plastic pellets (4–5 mm in diameter) made of polyethylene–vinyl acetate. These pellets were placed inside pellet holders (Fuji Flavor Co., Tokyo) and set 2 cm above the trap lip. Traps were positioned with the pheromone dispenser at 2.5 m above the ground. The candidate lures were replicated ($N = 2$) in randomized blocks with an intertrap distance of 10 m. Capture data were collected daily for at least three consecutive days and, after inspection, the traps were rerandomized. Capture data were transformed to log $(x + 1)$ before differences among means were tested for significance by ANOVA with JMP Software, Version 2 (Anonymous, 1989). Treatments followed by the same letters are not significantly different at the 5% level in the Tukey-Kramer honestly significant difference test.

RESULTS AND DISCUSSION

GC and GC-MS analyses of the crude extract from abdominal glands of *H. consanguinea* females gave a very clean profile (Figure 1). Some of the peaks appeared also in the solvent when it was concentrated to the same extent

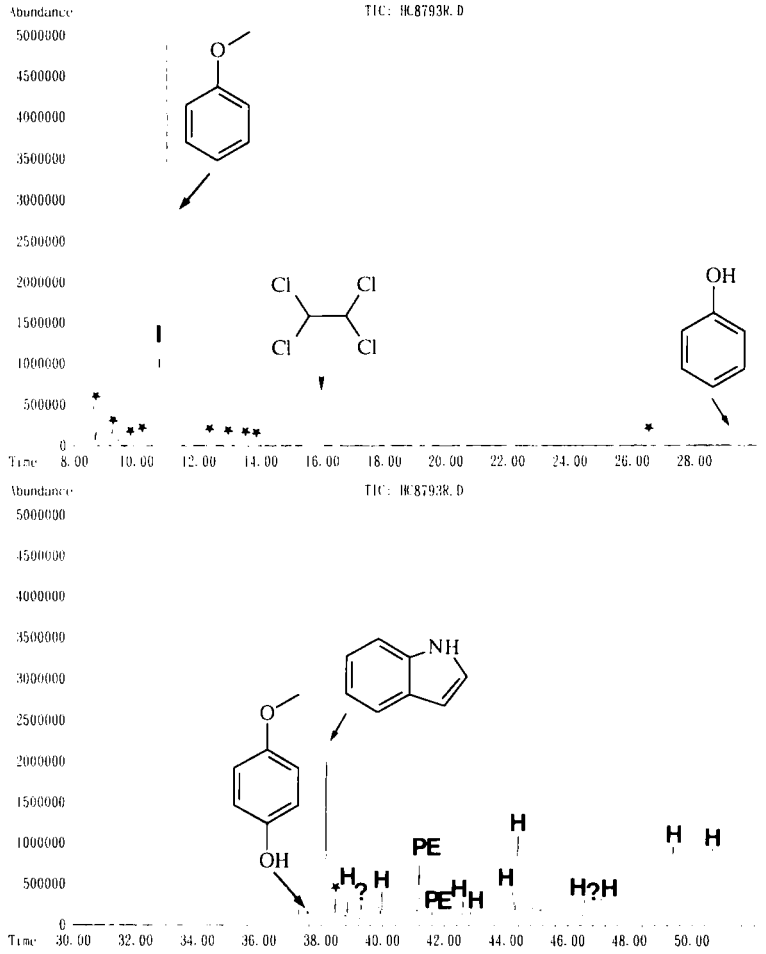

FIG. 1. Reconstructed total ion monitor profile of a crude extract sample from abdominal glands of *H. consanguinea* separated on a polar capillary column, HP-Innowax. The peaks labeled with an asterisk are due to solvent impurities. Cuticular hydrocarbons and phthalate esters are labeled with H and PE, respectively.

as the samples. One peak (I) was considered to be an impurity because it was observed only in one of 12 samples analyzed. Apart from the hydrocarbons, probably of cuticular origin, and phthalate esters, there were only a few relevant peaks. These were identified as methoxybenzene (anisole), 1,1,2,2-tetrachloroethane (an impurity from dichloromethane), phenol, 4-methoxyphenol, and indole.

Indoor bioassays revealed that the hexane–ether (80:20) fraction was very active. Males responded by attempting to take flight, orienting towards the pheromone source, aggregating on the source, and trying to copulate with each other. However, it was not possible to implement the bioassay for all the fractions under the same conditions. This was due to the fact that *H. consanguinea* is active for a very short period of time. Responses of one group to a certain fraction could not be compared to the responses of other individuals to a different fraction. This difficulty is intrinsic to the behavior of *Holotrichia* and *Phyllophaga* species. In order to overcome this barrier, bioassays to identify the active compound(s) in the pheromone system of *H. parallela* have been conducted in the field (Leal et al., 1992). This required a reasonable amount of sample and ultimately the full mixture could be identified only by assaying synthetic candidate compounds (Leal et al., 1993).

Because the indoor bioassay-oriented strategy could not lead unambiguously to the active compound(s) in a short period of time, we changed our strategy and assayed the insect response to candidate compounds in the field. The two major compounds found in the gland extracts, anisole and indole (Figure 1), were considered as possible attractants, notwithstanding the fact that indole is normally found in feces of scarab beetle males and females (Leal, unpublished data). Since phenol has been previously identified as a sex pheromone of another melolonthine species, *Costelytra zealandica* (Henzell and Lowe, 1970), it was also tested.

Field experiments showed that all the lures containing anisole were very attractive (Figure 2), whereas traps baited with indole and phenol (either alone or combined) did not capture significantly more beetles than control traps. Traps baited with caged virgin females did not capture significantly more than control traps, but this might be due to the fact that females of *Holotrichia* species hardly call when they are confined in a narrow cage (Leal, personal observation). Catches in traps baited at 3, 4.5, and 6 m were not significantly different.

Interestingly, the male-to-female ratio of the beetles captured was very close to 1:1. It has been previously observed that traps baited with synthetic sex pheromone of the Oriental beetle (and other species) also catch females (Leal et al., 1994). Nevertheless, the 1:1 ratio is very unusual. Recently, we have tested a previous hypothesis that males of the Oriental beetles caught in the traps released a semiochemical that would attract females. Traps baited with 30 males failed to attract any females in the field (Leal, unpublished data). On

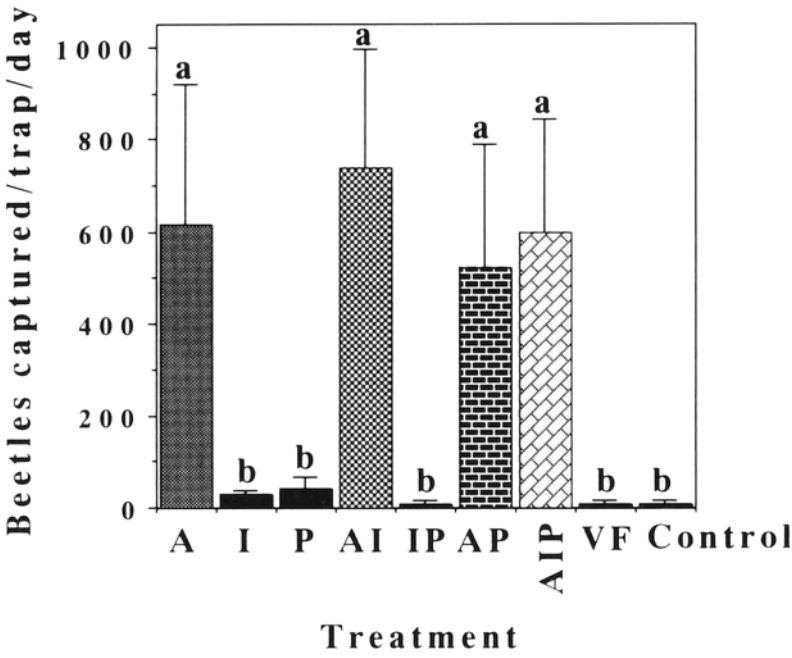

FIG. 2. Capture data of *H. consanguinea* in traps baited with candidate pheromones (A, anisole; I, indole; P, phenol), a combination of these compounds, virgin females (VF), and control traps.

the other hand, we also have evidence that female *Anomala octiescostata* can detect their own sex pheromone (Leal, unpublished data).

Recently, male-released pheromones have been identified from two scarab species in the subfamily Dynastinae, namely, *Oryctes monoceros* (Gries et al., 1994) and *O. rhinoceros* (Hallet et al., 1995). Because of male and female attraction to the semiochemicals, they were referred to as "aggregation" pheromones. However, there was evidence that chemical communication in *H. rhinoceros* involves a female-released sex pheromone in addition to the aggregation pheromone (Hallet et al., 1995).

In order to determine whether the male and female responses in *H. consanguinea* would be different during the calling period, we collected the capture data for both sexes at intervals of 5–10 min. Throughout the period of mating activity, the average ratio of three traps separated by 10 m was very close to 1 : 1 (Figure 3). Because the normal ratio in the field is also 1 : 1, we concluded that both males and females respond equally to the female-released compound.

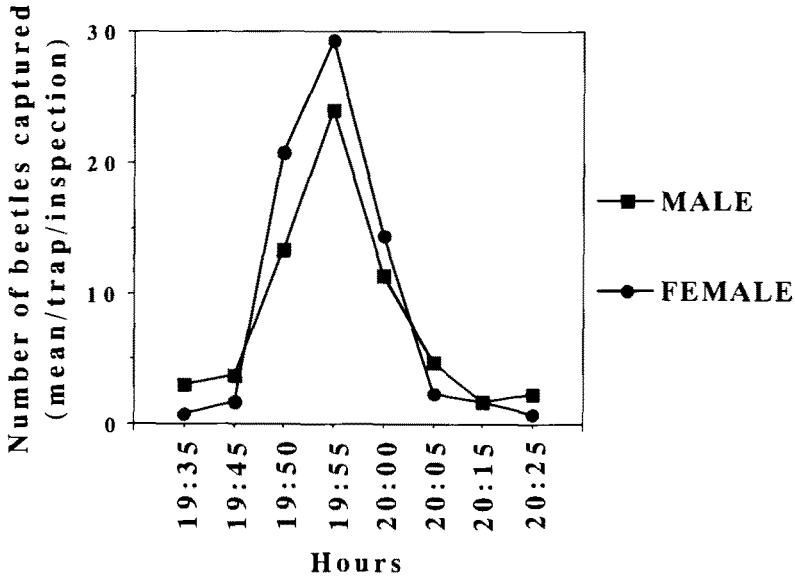

FIG. 3. Male and female responses of *H. consanguinea* as revealed by trap catches during the flight activity of July 12, 1995, in Durgapura. Throughout the period the male-to-female ratio was close to 1 : 1.

Sex pheromones have been considered by researchers to be those compounds that are emitted by individuals of one sex and that cause attraction of members of the opposite sex, resulting in the location of the emitter, and subsequently, mating (Baker, 1989; Tamaki, 1985; for a review of terminology, see also Nordlund, 1981). In that sense, anisole may be considered a sex pheromone because it is emitted by female beetles and males responded by displaying a clear sexual behavior. Males attracted to anisole tried to copulate with any individual (either male or female) in the vicinity. They also fluttered their wings when sitting on the trap lips or on the strings used to hang the traps. However, females were equally attracted to the same compound, and those that were not trapped displayed calling behavior on the traps.

The active compounds could be referred to as aggregation pheromones (sensu Borden, 1985): "substances that are produced by members of either and both sexes that induce members of both sexes to aggregate." It is not clear on an evolutionary basis why competing females are attracted to the female-released semiochemical. Thus, we rather prefer to refer to the semiochemical of *H. consanguinea* as a "female-released pheromone."

It is worth noting that aggregations of both sexes of melolonthines species

have been documented in other species as well. In the case of *H. serrata*, a species also native to India, it has been hypothesized that the chemical cues involved in the aggregation are not likely produced by only one of the two sexes to attract the opposite sex, because, if only females produce the cues to attract males and/or females, then the formation of pure male clumps could not be explained. Similarly, the formation of pure female clumps could not be explained if only males produced the pheromones to attract the males and/or females (Ganeshian and Kumar, 1993). We have no evidence for the formation of clumps of only one sex in *H. consanguinea*.

The adaptive basis of pheromone response by both sexes is not clear. It might not be selected for attracting females because it increases competition for mates. However, if the aggregation has any advantage other than mate finding, selection could be expected to favor the use of a pheromone that is not sex-specific. Due to our meager knowledge of the chemical ecology of this species, the advantage is obscure to us. It is intriguing, however, that such a phenomenon was found in a species that displays calling behavior in a very narrow window. Minimizing the period of calling and having, at the same time, a pheromone that attracts both males and females could be advantageous in escaping from a predator, for example.

These findings seem to shed more light on the secondary function hypothesis for the evolution of sex pheromonal communication in melolonthines (Leal, 1995). More recent findings support the conclusion that melolonthines largely utilize more "primitive" compounds as sex pheromones that may be also involved in other primary functions (Yarden et al., 1996; Leal et al., unpublished data), although one exception has been found. (*R*,*Z*)-7,15-Hexadecadien-4-olide has been identified as a sex pheromone of the Melolonthinae beetle *Heptophylla picea* (Leal et al., 1996).

Additional strong evidence for the secondary function hypothesis for the origin of sex pheromones in scarab beetles is the serendipitous discovery of the attraction of male *Cyclocephala lurida* (Scarabaeidae: Dynastinae) to grubs (Haynes et al., 1992). A recent study has been conducted (during a sabbatical leave of W.S.L. at Cornell University) to test whether the attraction to grubs was mediated by the same chemical(s) used by adults as sex pheromone. GC-EAD analyses of airborne volatiles and whole-body extracts from female beetles and whole-body extracts from grubs revealed that there is a common EAD-active component in the grubs and adult female extracts. That this EAD-active compound was involved in pheromonal communication was corroborated by a GC–behavioral bioassay (GC-BB). Males responded by gathering at the GC outlet soon after the EAD-active peak eluted from the GC, and some males displayed precopulatory behavior (Leal et al., unpublished data). The hypothesis for the ontogeny of the sex pheromone in this species is discussed elsewhere (Haynes and Potter, 1995).

If the chemical has preceded the evolution of functional communication in scarab beetles, it is possible that only later in the evolution a signal becomes sex-specific. *H. consanguinea*, for example, could be on an evolutionary ladder in which the signal is yet to be evolved as a sex-specific chemical cue.

We hope that this speculative hypothesis will stimulate further research on chemical communication of scarab beetles.

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STRUCTURE–ACTIVITY RELATIONSHIPS IN GLUCOSINOLATES AND OTHER COMPOUNDS STIMULATING OVIPOSITION IN THE CABBAGE ROOT FLY (*Delia radicum*)

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Abstract—The ability of a range of glucosinolates and other compounds in stimulating oviposition of the cabbage root fly (*Delia radicum*) was determined using a bioassay based on the use of surrogate leaves coated with test compounds. The results show that chemically dissimilar compounds are effective stimulants providing they contain the S=O group. No other part of the glucosinolate molecule was shown to be necessary. It has been shown that compounds other than glucosinolates may be important in the oviposition behavior of *D. radicum*.

Key Words—Oviposition, behavior, Insecta, Diptera, Anthomyiidae, *Delia radicum*, host plant chemicals, glucosinolates, sulfate, structure–activity relationships, bioassay.

INTRODUCTION

In the cabbage root fly, *Delia radicum* (L.) (Diptera, Anthomyiidae), oviposition behavior is associated with host plant location and recognition. The host plants are found mainly within the family Cruciferae and facets of behavior are influenced by host plant chemicals that are largely specific to that family (Hawkes et al., 1978). Before contact with the plant, isothiocyanates induce upwind

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orientation and increase flight activity in mated and gravid females (Hawkes and Coaker, 1979). The behavioral outcome is the movement upwind of females in a series of flights. On landing on the host plant, glucosinolates stimulate the first of a series of behavior patterns that result in eggs being laid generally in the soil in the vicinity of the host plant (Tranyier, 1967). Contact with glucosinolates or host plant material is necessary for oviposition, the presence of volatile isothiocyanates being insufficient alone (Nair and McEwen, 1976).

Electrophysiological studies (Roessingh et al., 1992) have shown that tarsal contact chemoreceptors occur in female cabbage root fly that are sensitive to glucosinolates. The biological activity of a range of glucosinolates was investigated using electrophysiological recording and a bioassay based on the use of surrogate leaves coated with test compounds. A significant correlation was found between the overall length of the side chain of the glucosinolate molecule and biological activity. The most stimulating fraction of plant surface extract contained no glucosinolates, and it was concluded that compounds in addition to glucosinolates play an important role in oviposition.

In this paper we report the results of an investigation into the structure–activity relationship of glucosinolates and other related compounds as oviposition stimulants for *D. radicum* using a bioassay method.

METHODS AND MATERIALS

Insects. All experiments and the culture of flies were carried out in a climate controlled room (18 L:6 D-photoperiod, 21 ± 1.5°C and 70 ± 10% relative humidity). A culture was maintained using the method of Finch and Coaker (1969).

For the bioassays, emerging flies were transferred into a separate holding cage every 24 hr and used in experiments when 7–8 days old.

Oviposition Sites. The design of the surrogate leaf structure was adapted from Roessingh and Städler (1990) and Koštál (1993). Each leaf consisted of a circle of white cardboard (9 cm diameter) with vertical folds 1 cm apart. This was slotted into a plastic tube stem (4 cm length, 0.5 cm diameter). The structure was then coated with a uniform layer of paraffin wax (Merck, mp 42–44°C) by a short immersion into warm water with a floating layer (0.6–1 cm) of the molten wax on top. Three leaves were sprayed with 0.6 ml of a 2.5×10^{-5} M solution of sinigrin in 75% aqueous methanol as the standards, three were treated with 0.6 ml of a test solution at a known concentration in 75% aqueous methanol, and the final three were treated with 75% aqueous methanol as the blanks. The model leaves were left to dry for approximately 30 min and then each one was fixed into the center of a Petri dish (9 cm diameter, 1.5 cm deep). Fine sand was placed into the dish around the leaf in which the flies could lay their eggs.

TEST 1	TEST 2	BLANK
BLANK	TEST 1	TEST 2
TEST 2	BLANK	TEST 1

FIG. 1. Layout of surrogate leaf structures on the bioassay cage floor. Test 1 is the control standard of leaves treated with 2.5×10^{-5} M sinigrin solution and test 2 is the compound under test.

The accuracy of the application technique in the preparation of the surrogate leaves was tested by recovery experiments using HPLC (Spinks et al., 1984). Sinigrin was recovered 95% or greater levels from the leaves in four repeat experiments.

Bioassays. Bioassay experiments were performed in screen cages (50 × 50 × 50 cm), on the floor of which were placed nine surrogate leaf structures in a random grid arrangement (Figure 1). Ten female flies, 7–8 days old, and an average weight of 11 mg, were introduced into the cage for 24 hr, after which times the "leaves" were removed and the number of eggs laid on and around each one determined by flotation in water.

Treatment of Results. The best-fit model of the observed data was found to be a cubic equation relating the fly oviposition response to the natural log of the concentration as shown:

$$Y = ax^3 + bx^2 + cx + d$$

where x is $\log_{10}$ concentration (molar), Y is relative stimulation $(T_2 - B)/(T_1 - B)$, with T_1 the total number of eggs laid on the sinigrin control standards (test 1), T_2 the total number of eggs laid on the "leaves" treated with the test compound (test 2), and B the total number of eggs laid on the blanks.

The optimum concentration (C) of a compound required to give maximum relative stimulation (Y_{max}) for each compound was then calculated from the model and used as a measure of biological activity. Compounds were designated as biologically active if egg laying on the treated sites was significantly higher than that on the blanks according to the F test for variance and the appropriate t test. A value of Y greater than 0.01 was obtained as the level indicating significant oviposition stimulation.

Test Compounds and Their Synthesis. All test compounds and the starting materials were purchased from Aldrich UK Ltd. and used as received unless stated otherwise. Potassium salts of three different glucosinolates were used: sinigrin, glucobrassicin, and glucotropaeolin. Sinigrin was purchased from Sigma UK Ltd., and the other two were provided by the Department of Biological

Sciences, University of Plymouth. Desulfosinigrin, sinigrin tetraacetate, acetone sodium bisulfite, *S*-methyl cysteine sulfoxide, and the corresponding sulfone were synthesized as described below.

Desulfosinigrin was obtained by the desulfation of the glucosinolate molecule using the enzyme aryl sulfatase (Sigma UK Ltd.). The enzyme (10 mg) was added to 5 mg of sinigrin dissolved in 50 ml of 0.2 M acetate buffer, pH 5.57. The solution was left overnight at 25°C and the desulfation was considered to be complete since the sample was chromatographically homogeneous following the procedure of Spinks et al. (1984) with no trace of sinigrin present. The reaction solution was bioassayed immediately with an appropriate blank.

Sinigrin tetraacetate was synthesized according to the method of Kjaer and Gmelin (1956). A 10-fold excess of acetic anhydride was used, and the reaction mixture was kept at 50°C for 6 hr. The product had a melting point of 194°C (lit. value 194°C; Benn and Ettlinger, 1965). The material was chromatographically homogeneous as shown by thin-layer chromatography. R_f values of 0.27 and 0.6 were obtained on 60 G silica gel plates for sinigrin and its tetraacetate, respectively, with an *n*-butanol–acetic acid–water (60 : 15 : 25% by volume) solvent system.

The bisulfite addition compound was prepared according to Vogel (1970) and *S*-methyl cysteine sulfoxide according to Greenstein and Winitz (1961). *S*-Methyl cysteine sulfone was synthesised by the oxidation of *S*-methyl cysteine sulfoxide.

RESULTS

Table 1 shows the structures, optimum concentrations (C), and maximum stimulation relative to the 2.5×10^{-5} M sinigrin standard (Y_{max}) for three naturally occurring glucosinolates and two structural modifications of sinigrin. Table 2 shows similar data for 12 other nonglucosinolate compounds tested, plus one mixture.

Figures 2–4 show plots of relative stimulation (Y) versus $\log_{10}$ concentration (x) for all of the compounds tested together with the best-fit curves in each case from which C and Y_{max} can be calculated.

Glucobrassicin (Table 1, compound 1) proved to be the most effective compound in terms of number of eggs laid relative to a standard (Y_{max}). Examination of Tables 1 and 2 together with Figures 2–4 shows that the concentration of a compound (C) required to produce the maximum stimulus (Y_{max}) shows a considerable range (4.29×10^{-4} to 1.16×10^{-6} M). All of the bioactive compounds show a decrease in activity at higher concentrations.

Figure 5 shows a plot of maximum relative stimulation (Y_{max}) against optimum concentration (C) for each compound. With the exception of sinigrin and

TABLE 1. NAMES, STRUCTURES, AND DETAILS OF ACTIVITY OF NATURAL AND MODIFIED GLUCOSINOLATES BIOASSAYED

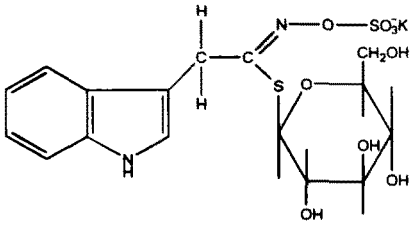
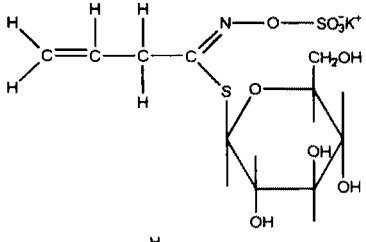
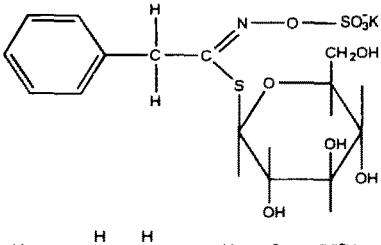
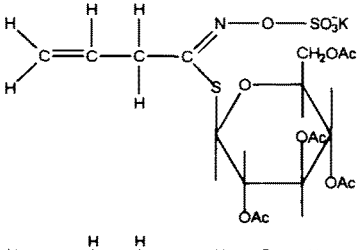
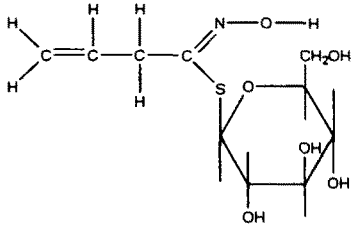
No.	Structure	Compound name	C	Y_{max}
1		Glucobrassicin	1.4×10^{-5}	5.30
2		Sinigrin	3.8×10^{-4}	2.82
3		Glucotropaeolin	2.2×10^{-6}	1.83
4		Sinigrin tetraacetate	5.5×10^{-5}	1.17
5		Desulfated sinigrin	Not active	Not active

TABLE 2. NAMES, STRUCTURES, AND DETAILS OF ACTIVITY OF NONGLUCOSINOLATE TEST COMPOUNDS BIOASSAYED

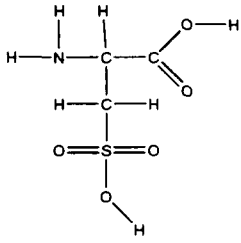
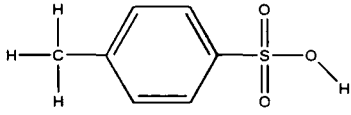
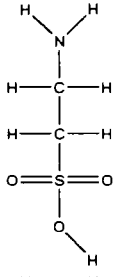
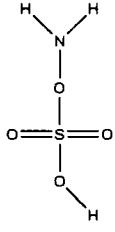
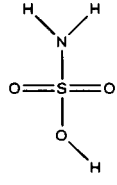
No.	Structure	Compound name	C	Y_{max}
1		Cysteic acid	1.97×10^{-6}	1.57
2		*p*-Toluene sulfonic acid	8.36×10^{-5}	1.10
3		Taurine	1.16×10^{-6}	1.63
4		Hydroxylamine-*O*-sulfonic acid	4.39×10^{-5}	1.67
5		Sulfamic acid	4.99×10^{-5}	2.01

TABLE 2. CONTINUED

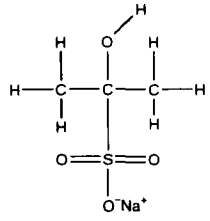
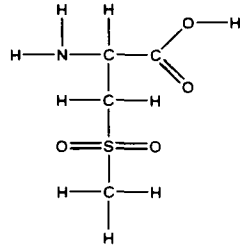
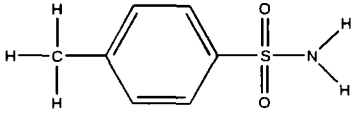
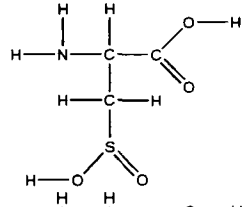
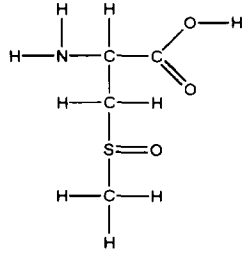
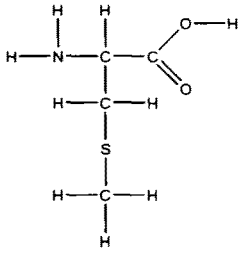
No.	Structure	Compound name	C	Y_{max}
6		Acetone sodium bisulfite	4.29×10^{-4}	0.7
7		*S*-Methyl cysteine sulfone	4.11×10^{-6}	1.37
8		*p*-Toluene sulfonamide	1.37×10^{-6}	1.12
9		Cysteine sulfinic acid	1.59×10^{-6}	1.28
10		*S*-Methyl cysteine sulfoxide	1.44×10^{-6}	1.16

TABLE 2. CONTINUED

No.	Structure	Compound name	C	Y_{max}
11		*S*-Methyl cysteine	Not active	Not active
12	$NaHSO_4$	Sodium bisulfate	Not active	Not active

glucobrassicin, all the bioactive compounds lie in a narrow range of Y_{max} values (between 1 and 2). Compounds with the low optimum concentrations have high biological activity but the high relative stimulation of glucobrassicin also indicates high activity.

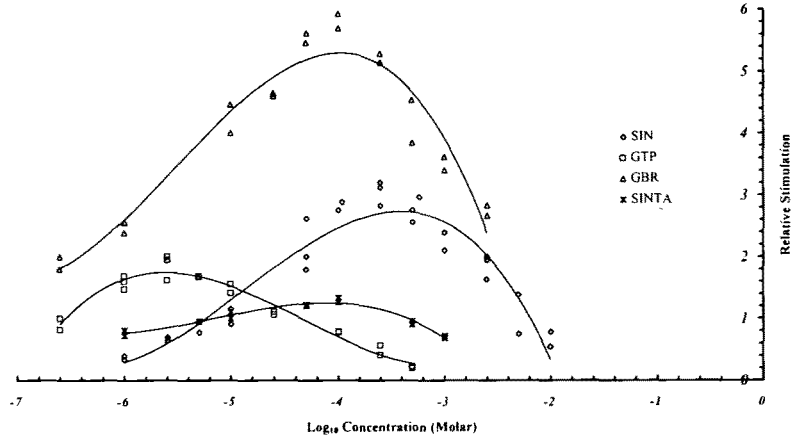


FIG. 2. Bioassay data and regression lines for sinigrin (SIN), glucotropaeolin (GTP), glucobrassicin (GBR), and sinigrin tetraacetate (SINTA).

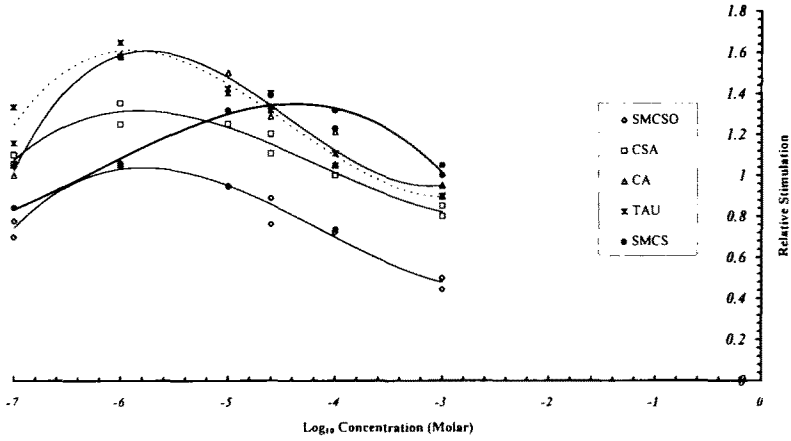


FIG. 3. Bioassay data and regression lines for *S*-methyl cysteine sulfoxide (SMCSO), cysteine sulfinic acid (CSA), cysteic acid (CA), taurine (TAU), and *S*-methyl cysteine sulfone (SMCS).

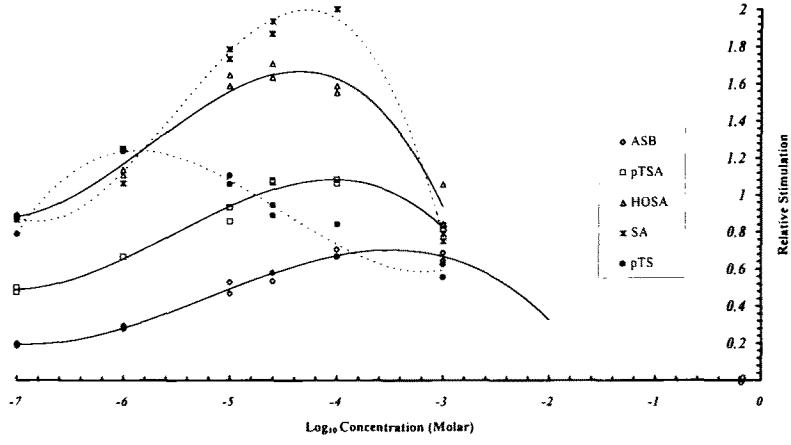


FIG. 4. Bioassay data and regression lines for acetone sodium bisulfite (ASB), *p*-toluene sulfonic acid (pTSA), hydroxylamine-*O*-sulfonic acid (HOSA), and *p*-toluene sulfonamide (pTS).

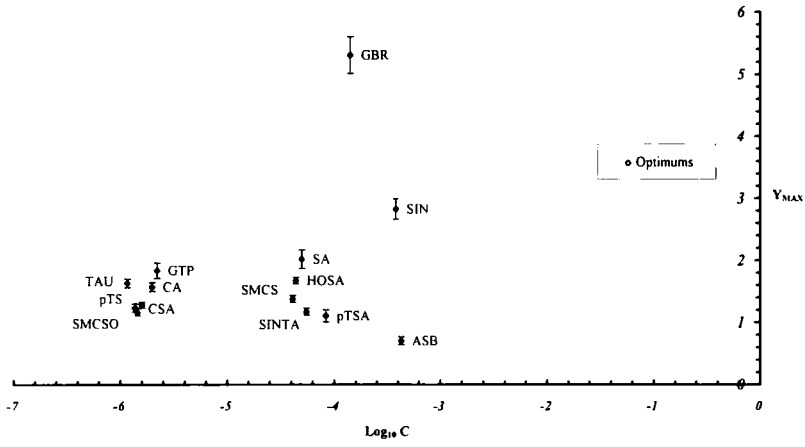


FIG. 5. Optimum concentrations and responses for all active test compounds.

DISCUSSION

The naturally occurring glucosinolate molecules can be divided into three structural regions: a side-chain (R), a thioglucose, and a sulfated oxime. Examination of Table 1 shows that variations in R result in modification of activity, demonstrated by sinigrin, glucobrassicin, and glucotropaeolin. Similar findings have been reported by Roessingh et al. (1992) for *D. radicum* and by Simmonds et al. (1994) for *D. floralis*.

Modification of the thioglucose residue of sinigrin by acetylating all of the hydroxyl groups to give sinigrin tetraacetate caused some decrease in activity. The level was well above the threshold of nonstimulus, however. This implies that the glucose residue is not a key structural requirement.

Desulfation of the oxime sulfate group resulted in the formation of desulfated sinigrin, which was devoid of all activity. The sulfate group seems to be an essential structural feature for oviposition—a region that could be identified as an on–off switch for egg laying.

A series of compounds containing the SO_3H group bonded to a variety of other organic residues or functional groups was then examined to test this hypothesis. Table 2, compounds 1–6, shows that all the compounds examined that contained the SO_3H group possessed significant biological activity despite the wide variety of structures attached to the group. Toluene-*p*-sulfonic acid (2) was tested as both a free sulfonic acid and as the potassium salt. There was no difference in the bioassay results, showing that it is not necessary to have the ionized SO_3^- group rather than the parent acid. The most effective compound

in terms of Y_{max} was the simple compound sulfamic acid (5), in which the sulfur atom is bonded directly to an amino-nitrogen atom. Hydroxylamine-*O*-sulfonic acid (4), which contains the three atom system N-O-S present in glucosinolates, was also an effective stimulant.

Attempts to sulfate the oxime group of simple ketones to produce compounds more strictly analogous to glucosinolates were unsuccessful, the reaction producing an amide by a spontaneous Beckmann rearrangement of the desired product. This result was not unexpected.

The results obtained from compounds showing a progressive decrease in the oxidation state of the original SO_3H sulfur atom are given in Table 2, compounds 6–11. From these results it becomes quite clear that the key structural requirement for oviposition stimulus is the presence of an S=O group in a molecule. This group is present in *S*-methyl cysteine sulfoxide (10), which was bioactive. *S*-Methyl cysteine, lacking the S=O group, was devoid of biological activity. Sodium bisulfate (12), in which the S atom is in its maximum oxidation state, also lacked activity.

Städler and Shöni (1990) produced evidence that more than one group of compounds stimulates oviposition. Roessingh et al. (1992) report that compounds additional to glucosinolates play a role in the stimulation of oviposition. The compounds were not identified, however.

S-Methyl cysteine sulfoxide (10) is known to be present in the leaves of a variety of Brassicae, including cabbage at significant levels (Morris and Thompson, 1956; Synge and Wood, 1956). In view of the demonstration of its oviposition-stimulating ability on surrogate leaves, it is possible that it could have the same property in vivo. However there was no synergistic activity between sinigrin and (10) (Table 3), the effect being only additive.

TABLE 3. RESULTS OF SYNERGISM EXPERIMENTS WITH SINIGRIN AND *S*-METHYL CYSTEINE SULFOXIDE

Compounds	Initial concentration (M)	% Used in test mixture	Concentration in test mixture (M)	Expected *Y* (additive)	Actual *Y*
Sinigrin +	1×10^{-5}	90	9×10^{-6}	2.31	2.00
S-methyl cysteine sulfoxide	1×10^{-4}	10	1×10^{-5}		
Sinigrin +	1×10^{-4}	50	5×10^{-6}	1.78	1.72
S-methyl cysteine sulfoxide	1×10^{-4}	50	5×10^{-5}		
Sinigrin +	1×10^{-5}	10	1×10^{-6}	1.05	0.98
S-Methyl cysteine sulfoxide	1×10^{-4}	90	9×10^{-5}		

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CALCIUM CHLORIDE AS A MAJOR COMPONENT CONTRIBUTING TO APHID RESISTANCE OF *Nicotiana benthamiana*

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Abstract—Substances with antiaphid activity were extracted from the leaf surface of aphid-resistant *N. benthamiana* and purified. Sugar esters and diacyl glycerols were isolated from the chloroform extract, but only diacyl glycerols showed significant activity. However, the major activity was found in the water extract rather than in the chloroform extract. From the water extract calcium chloride was isolated as the most abundant active substance. Only calcium chloride showed significant activity among several calcium salts and chlorides of several metals that are abundant in plants. Calcium contents per unit area of leaf surface of *N. benthamiana* and aphid-resistant *N. gossei* were almost equal to each other and 10–100 times higher than that of aphid-susceptible *N. tabacum*.

Key Words—*Nicotiana benthamiana*, *Myzus persicae*, green peach aphid, *Nicotiana gossei*, calcium chloride, plant resistance to insects.

INTRODUCTION

Aphids damage a wide range of crops not only by feeding on them but also by spreading viruses from one plant to another. Various kinds of pesticide have

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been developed to control aphids. However, they are not always able to control aphids since the pests multiply very quickly and often acquire immunity to pesticides. Several plant species are known to resist aphids. Understanding the resistance mechanisms could suggest ways to develop safe and effective pesticides as well as ways to breed new aphid-resistant varieties. *Nicotiana benthamiana* is highly resistant to aphids (Hagimori et al., 1993) and the leaf-eating caterpillar *Spodoptera litura* (Joshi and Sitaramaiah, 1975). Somatic hybrids between *N. benthamiana* and *N. tabacum* have already been produced to introduce aphid resistance into tobacco (Hagimori et al., 1993). In the course of this work, we observed numerous droplets—considered to be trichome exudates—on the leaf surface of *N. benthamiana*. The aphid resistance of *N. benthamiana* leaf explants decreased to the level of *N. tabacum* if the droplets were removed, suggesting that the causative agent of the resistance is present in the droplets. The purpose of the present work is to isolate and identify substances with antiaphid activity present on *N. benthamiana* leaf surfaces. Leaf surface lipids, especially sugar esters, were attributed to be the cause of the aphid resistance of several plant species (Goffreda et al., 1989; Neal et al., 1990; Severson et al., 1992). In *N. benthamiana*, we previously analyzed leaf surface lipids and found novel glycerolipids and glycolipids (Matsuzaki et al., 1992). Therefore, we first examined these lipids for antiaphid activity. We subsequently searched for other active substances in the leaf surface extract of *N. benthamiana*.

METHODS AND MATERIALS

Plant Materials. *Nicotiana benthamiana* Domin., *N. gossei* Domin. (both of which are aphid resistant), and *N. tabacum* L. (which is aphid susceptible) were used in this work. They were grown in a greenhouse and were watered carefully so as not to wet their leaves to avoid washing off leaf surface substances.

Analysis of Leaf Surface Lipids. Leaves of 3-month old plants of *N. benthamiana* were collected. The leaf surface lipids were extracted by dipping the leaves into chloroform for 10 sec. The chloroform-extracted lipids were separated by silica gel column chromatography and high-performance liquid chromatography (HPLC) according to Matsuzaki et al. (1992).

Extraction and Fractionation of Leaf Surface Substances. Leaves of 3-month-old plants of *N. benthamiana* were collected. Leaf surface material was extracted with either chloroform or distilled water by dipping the leaves in the solvent for 20 sec. Both chloroform and water extracts were assayed for antiaphid activity. The water extract was designated WS. A portion of WS was extracted with chloroform twice. The combined chloroform extracts were designated WS1. The residue was dissolved in water and designated WS2. Both

WS1 and WS2 were assayed for antiaphid activity. Diaion HP20 was used to fractionate WS2 by ion exchange chromatography into three fractions, HP1, HP2, and HP3. First, HP1 was obtained by eluting with water, then HP2 and HP3 were eluted successively with 20% ethanol and ethanol, respectively. HP1 was further fractionated by HPLC using the Shodex KS801 column. The solvent used in the HPLC was water, and the flow rate was 1 ml/min. Effluent was scanned with a refractive index photometer. Only one peak was detected. The fraction of the peak was collected and designated P1.

Antiaphid Activity Assay. A colony of green peach aphids, *Myzus persicae* Sulzer, was reared and maintained on tobacco seedlings in a greenhouse at 22°C. Apterous viviparous adults were collected and starved for 4 h before use. Two 96-well microplates for ELISA (Nunc) were used for each assay. In one microplate, one aphid was deposited in each of the 96 wells. In another microplate, either 400 μl of 20% sucrose solution or 400 μl of artificial diet, which contains sucrose, amino acids, vitamins, and minerals (Mittler and Koski, 1974), was placed in each of the 96 wells as food for the aphids, and the microplate was sealed tightly with stretched Parafilm (American National Can, Greenwich, Connecticut). A 20% sucrose solution was used in the assays for the activity of the leaf surface lipids and the activities of the crude extracts, WS, WS1, and WS2. The artificial diet, which is thought to be the more suitable food for aphid, was used in the other assays except for the assay of the toxicity of calcium chloride. In the assay of the toxicity, no aphid food was supplied. The substance to be assayed was dissolved in either methanol or distilled water and spread on the upper surface of the Parafilm at a concentration 100 $\mu g/cm^2$. For the control, either methanol or distilled water was spread on the upper surface of the Parafilm. After the surface dried, one microplate sealed with the Parafilm was placed upside down on top of another one that contained aphids so that the wells of the two microplates faced each other with the Parafilm held between them. The microplate assembly was turned over to make the aphids settle on the Parafilm. Then the assembly was turned over again and incubated at 25°C in darkness for 72 h. Every 24 h we counted the number of aphids that settled on the Parafilm and the number of unsettled living and dead ones.

Identification of P1. X-ray analysis was performed using a JSM-6400 X-ray analyzer equipped with SEM-EDX (JEOL Ltd., Tokyo, Japan). ^{1}H NMR spectra were obtained with a Bruker AC-300 NMR spectrometer. HPLC analysis was performed using an ODS column (YMC pack A321). Water was used as the solvent at a flow rate of 1 ml/min.

Quantitative Analysis of Calcium on Leaf Surface. About 10 g of fresh leaves were collected from each individual. The total area of the leaves was measured by the AAC-400 (Hayashi Denkoh Co., Ltd., Tokyo, Japan) automatic area meter. The leaves were dipped in 300 ml of deionized distilled water for 20 sec. The extract was evaporated in vacuo and residue was dissolved in

25 ml deionized distilled water. The calcium content in the solution was assayed by the ICPA-575 II (Nippon Jarrell-Ash, Uji, Japan) inductively coupled argon plasma atomic emission spectrophotometer.

Detection of Nicotine. Nicotine was detected by silica gel thin-layer chromatography. Two kinds of solvent were used: a 1 : 1 solution of hexane–acetone and a 65 : 25 : 4 solution of chloroform–methanol–water. Spots of nicotine were detected by spraying with Dragendorff's reagent.

RESULTS

Antiaphid Activity of N. benthamiana Leaf Surface Lipids. We obtained 60 g of leaf surface lipids from 10 kg of fresh *N. benthamiana* leaves. The leaf surface lipids were further fractionated to yield 1.2 g of 1,2-diacyl glycerol (1-*O*-acetyl-2-*O*-acylglycerol, DG), 12 g of glucose diester (3,4-di-*O*-acyl-α-D-glucopyranose, GE), 8 g of sucrose triester [6-*O*-acetyl-4-*O*-acyl-α-D-glucopyranosyl-3-(*O*-acyl)-β-D-fructofuranoside, STE], and 0.8 g of sucrose diester [4-*O*-acyl-α-D-glucopyranosyl-(3-*O*-acyl)-β-D-fructofuranoside, SDE]. These substances were assayed for antiaphid activity. Table 1 shows the results observed 48 h after initiation of the assay. GE, STE, and SDE had no effect on the aphids. Only DG showed significant activity of repelling and killing aphids.

Antiaphid Activity of Crude Extracts. Leaf surface substances of *N. benthamiana* were extracted with two kinds of solvents (i.e., water and chloroform). The crude extracts were assayed for antiaphid activity (Table 2). Chloroform

TABLE 1. ANTIAPHID ACTIVITY OF LEAF SURFACE LIPIDS OF *Nicotiana benthamiana*

	Number of aphids[b]			
Lipids[a]	Tested	Settling	Nonsettling	Dead
Diacylglycerol	50	9	19	22[c]
Control	50	24	23	3
Glucose dieseter	50	27	23	0
Control	50	35	15	0
Sucrose triester	50	22	28	0
Control	50	27	23	0
Sucrose diester	50	17	33	0
Control	50	15	31	0

[a] Each lipid was applied to the Parafilm membrane at 100 $\mu g/cm^2$; 20% sucrose solution was used as the aphid food.

[b] The number of aphids was counted 48 hr after initiation of the assay.

[c] Significantly different from control by chi square test ($P < 0.001$).

TABLE 2. ANTIAPHID ACTIVITY OF CRUDE EXTRACTS FROM *Nicotiana benthamiana* LEAF SURFACE EXTRACTED WITH CHLOROFORM AND WATER

Extracts[a]	Number of aphids[b]			
	Tested	Settling	Nonsettling	Dead
Chloroform	30	16	14	0
Control	30	6	23	1
Water	30	13	11	6[c]
Control	30	16	14	0

[a]Each extract was applied to the Parafilm membrane at 100 $\mu g/cm^2$; 20% sucrose solution was used as the aphid food.
[b]The number of aphids was counted 24 hr after initiation of the assay.
[c]Significantly different from control by chi square test ($0.01 > P > 0.001$).

extracts had no effect on the aphids. On the other hand, the water extract showed significant activity. This result demonstrates that major antiaphid substances of *N. benthamiana* are soluble in water and insoluble in chloroform.

Purification and Identification of Active Antiaphid Substances in Water Extracts of N. benthamiana Leaf Surface Substances. The water extract from *N. benthamiana* leaf surface substances was further fractionated to purify the active antiaphid substances. Figure 1 shows the procedure schematically and the yields of each fraction. The antiaphid activity of each fraction was assayed. In the first step, the water extract (WS) was separated into two fractions: WS1 and WS2. We obtained 410 mg WS1 and 5260 mg WS2 from 1.23 kg of fresh leaves. Thin-layer chromatography detected nicotine only in WS1, not in WS2. Table 3 depicts the antiaphid activity of WS, WS1, and WS2. Antiaphid activity in WS was concentrated in WS2, not in WS1. WS2 was further fractionated to obtain three fractions: 3.61 g HP1, 80 mg HP2, and 60 mg HP3. All of these fractions exhibited almost equally robust antiaphid activity (Table 4). HP1, by far the most abundant fraction, was subsequently subjected to HPLC using the Shodex column. Only one peak was detected, and the fraction was collected (P1, 2.86 g). P1 was subjected to further HPLC using an ODS column and, again, only one peak was detected.

Identification of P1. X-ray analysis of P1 showed the existence of calcium and chlorine. ^1H NMR of P1 gave no clear signals except for a broad singlet at 4.4 ppm due to H_2O. These results suggested that P1 was $CaCl_2 \cdot nH_2O$. The retention time of $CaCl_2 \cdot 2H_2O$ was the same as that of P1 in HPLC in both columns mentioned above. The antiaphid activity of $CaCl_2 \cdot 2H_2O$ was compared with that of P1. $CaCl_2 \cdot 2H_2O$ exhibited almost the same activity as P1 (Table 5). From these results, we concluded that P1 was $CaCl_2 \cdot 2H_2O$. The antiaphid

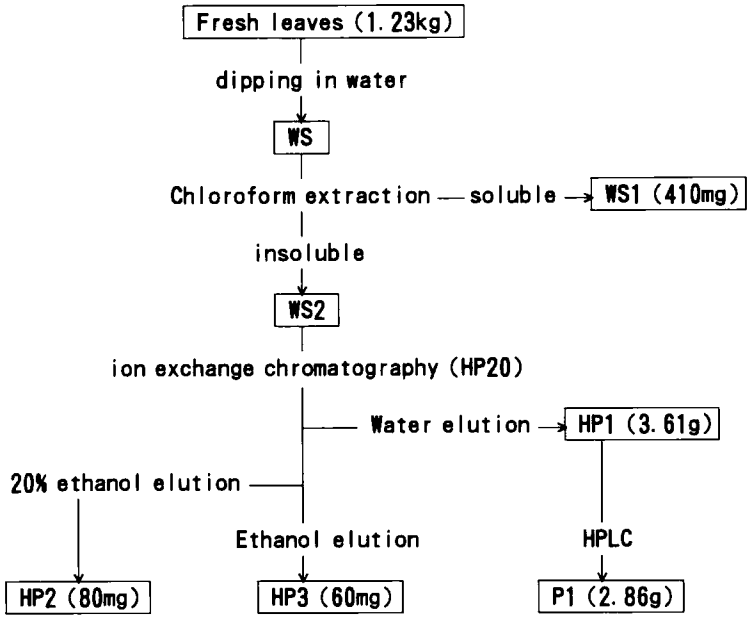

FIG. 1. Scheme of the isolation procedure of antiaphid active substances from fresh leaves of *Nicotiana benthamiana*.

activity of calcium chloride concentration, in the range of 5–1000 $\mu g/cm^2$, was examined. Calcium chloride showed significant activity at more than 50 $\mu g/cm^2$, but the levels of activity did not go up in response to increase in concentration higher than 50 $\mu g/cm^2$ (data not shown).

TABLE 3. ANTIAPHID ACTIVITY OF WATER EXTRACT (WS) FROM LEAF SURFACE OF *Nicotiana benthamiana* AND ITS FRACTIONS, WS1 AND WS2

Sample[a]	Number of aphids[b]			
	Tested	Settling	Nonsettling	Dead
WS	90	4	20	66[c]
WS1	90	40	36	14
WS2	90	6	23	61[c]
Control	90	36	40	14

[a] Each sample was applied to the Parafilm substrate at 100 $\mu g/cm^2$; 20% sucrose solution was used as the aphid food.
[b] The number of aphids was counted 48 h after initiation of the assay.
[c] Significantly different from control by chi square test at ($P < 0.001$).

TABLE 4. ANTIAPHID ACTIVITY OF FRACTIONS OF WS2 SEPARATED BY ION EXCHANGE CHROMATOGRAPHY (HP20)

Fraction[a]	Number of aphids[b]			
	Tested	Settling	Nonsettling	Dead
HP1	60	24	12	24[c]
HP2	60	17	17	26[c]
HP3	60	12	16	32[c]
Control	60	24	30	6

[a]Each sample was applied to the Parafilm membrane at 100 $\mu g/cm^2$. The artificial diet was used as the aphid food.
[b]The number of aphids was counted 74 hr after initiation of the assay.
[c]Significantly different from control by chi square test ($P < 0.001$).

Antiaphid Activity of Calcium Salts and Chlorides. To clarify which component of $CaCl_2 \cdot 2H_2O$ was responsible for the antiaphid activity, the following calcium salts and chlorides were assayed for the activity (Table 6): $CaCl_2 \cdot 2H_2O$, $Ca(H_2PO_4) \cdot 2H_2O$, $CaSO_4 \cdot 2H_2O$, $CaCO_3$, NaCl, KCl, and $MgCl_2$. Contrary to expectation, only $CaCl_2 \cdot 2H_2O$ showed significant activity. $MgCl_2$ may possess slight activity.

Calcium Content on Leaf Surface of N. benthamiana and Other Nicotiana *Species.* To clarify whether there is a correlation between aphid resistance and calcium content on the leaf surface, *N. benthamiana*, *N. gossei* (which is the most resistant among *Nicotiana*), and susceptible *N. tabacum* were assayed for calcium content on their leaf surface (Table 7). The calcium contents of the two resistant species, *N. benthamiana* and *N. gossei*, were nearly equivalent and were 10–100 times higher than that of susceptible *N. tabacum*.

TABLE 5. ANTIAPHID ACTIVITY OF FRACTION P1 ISOLATED FROM *Nicotiana benthamiana* EXTRACT AND CALCIUM CHLORIDE

Sample[a]	Number of aphids[b]			
	Tested	Settling	Nonsettling	Dead
P1	60	22	5	33[c]
$CaCl_2 \cdot 2H_2O$	60	25	7	28[c]
Control	60	38	14	8

[a]Each sample was applied to the Parafilm membrane at 100 $\mu g/cm^2$. The artificial diet was used as the aphid food.
[b]The number of aphids was counted 72 hr after initiation of the assay.
[c]Significantly different from control by chi square test ($P < 0.001$).

TABLE 6. ANTIAPHID ACTIVITY OF CALCIUM SALTS AND CHLORIDES

Salt[a]	Number of aphids[b]			
	Tested	Settling	Nonsettling	Dead
$CaCl_2 \cdot 2H_2O$	30	6	5	19[c]
$Ca(H_2PO_4)_2 \cdot H_2O$	30	13	10	7
$CaSO_4 \cdot 2H_2O$	30	15	10	5
$CaCO_3$	30	9	14	7
NaCl	30	16	9	5
KCl	30	13	10	7
$MgCl_2$	30	8	7	15
Control	30	10	11	9

[a] Each salt was applied to the Parafilm membrane at 100 $\mu g/cm^2$. The artificial diet was used as the aphid food.
[b] The number of aphids was counted 48 hr after initiation of the assay.
[c] Significantly different from control by chi square test ($0.01 > P > 0.001$).

Toxicity of Calcium Chloride to Aphids. To determine the mode of action of calcium chloride (whether it is a feeding deterrent or a toxic compound), calcium chloride was assayed for antiaphid activity without supplying aphid food. Calcium chloride showed significant activity also in this condition (Table 8).

DISCUSSION

Quite a few plant species are known to be aphid resistant. The mechanism of the resistance has been investigated, and several compounds existing in or

TABLE 7. *Nicotiana benthamiana*, *N. gossei*, AND *N. tabacum* LEAF SURFACE CALCIUM CONCENTRATIONS

Individual number	*Nicotiana* species	Sampled area (cm^2)	Ca concentration ($\mu g/cm^2$)
1	*benthamiana*	450	3.0
2	*benthamiana*	446	2.6
3	*benthamiana*	516	2.4
4	*gossei*	377	2.6
5	*gossei*	485	3.0
6	*gossei*	346	3.6
7	*tabacum*	447	0.06
8	*tabacum*	391	0.03
9	*tabacum*	321	0.36

TABLE 8. ANTIAPHID ACTIVITY OF CALCIUM CHLORIDE WITH NO APHID FOOD SUPPLIED

Sample[a]	Number of aphids[b]			
	Tested	Settling	Nonsettling	Dead
$CaCl_2 \cdot 2H_2O$	60	3	17	40[c]
Control	60	6	49	5

[a] $CaCl_2$ was applied to the Parafilm membrane at 100 $\mu g/cm^2$. No aphid food was used.
[b] The number of aphids was counted 24 hr after initiation of the assay.
[c] Significantly different from control by chi square test ($P < 0.001$).

on the surface of the plants were reported to be the cause of the resistance (Pickett et al., 1992; Corcuera, 1993). Sugar esters are among such compounds. Goffreda et al. (1989) attributed the aphid resistance of *Lycopersicon pennellii* to glucose esters of branched-chain carboxylic acids contained in trichome exudates of the plant. Aphid resistance of *Solanum berthaultii* was attributed to the 3′,3,4,6-tetra-*O*-acyl sucroses present in large amounts in the trichome exudates (Neal et al., 1990). In *Nicotiana gossei*, the most resistant species in the genus *Nicotiana*, Severson et al. (1992) reported that two types of glucose ester and two types of sucrose ester isolated from the cuticular extract were toxic to the tobacco aphid and the sweet potato whitefly. In the case of *N. benthamiana*, however, although sugar esters were present on the leaf surface in considerable amounts, they did not show the antiaphid activity.

In *Nicotiana* spp., nicotine alkaloids such as nicotine, nornicotine, anabasine, and *N*-acylnornicotines were attributed to be the cause of the resistance (Thurston et al., 1961; Huesing et al., 1989). In *N. benthamiana*, however, this is unlikely since nicotine was collected in the inactive fraction (WS1).

In this work, diacyl glycerol (DG) and calcium chloride were identifed as the active antiaphid substances present on the leaf surface of *N. benthamiana*. DG is not present on the leaf surface of susceptible *N. tabacum* (Hagimori et al., 1993), supporting the view that DG might be responsible for at least a portion of the resistance of *N. benthamiana* to aphids. However, the somatic hybrids between *N. benthamiana* and *N. tabacum* did not contain DG but were nevertheless almost as resistant to aphids as *N. benthamiana* (Hagimori et al., 1993). Furthermore, major antiaphid activity was in the water extract, not in the chloroform extract (Table 2), while DG is washed out from the leaf surface with chloroform. These results suggest that DG is not the major cause of the resistance.

It is generally agreed that for any plant substance to be regarded as the causative agent of resistance to aphids, it should meet the following criteria: First, the substance should have some antiaphid activity. Second, the concen-

tration of the substance in the part of the plant where the aphids feed should be high enough to cause the effects in artificial diets. Third, there should be a correlation between the concentration of the substances and plant resistance to the aphids (Corcuera, 1993). Our results indicate that calcium chloride meets the first and third criteria, while it does not appear to meet the second. The concentration of calcium on the leaf surface of *N. benthamiana* and *N. gossei* was around 3 $\mu g/cm^2$. This calcium value is equivalent to about 11 μg of $CaCl_2 \cdot 2H_2O$. This is still only one fifth of the minimum effective concentration used in the antiaphid activity assay (50 $\mu g/cm^2$). Although it is not known how the calcium chloride comes to exist on the leaf surface, it is unlikely to reside uniformly on the leaf surface as was the case with the Parafilm membrane used in the assay. It is more likely to be concentrated around the exuding tissues on the leaf, such as the trichomes or stomata, to a level high enough to repulse aphids. Thus, it is possible that the second criterion might also be fulfilled.

Other unidentified active substances were indicated to be in fractions HP2 and HP3. Their activities were comparable to that of calcium chloride at the concentration of 100 $\mu g/cm^2$, but their concentrations on the leaf surface of *N. benthamiana* were much lower than that of calcium chloride.

The antiaphid activity levels were almost the same when concentrations were more than 50 $\mu g/cm^2$. It seemed that the concentration was saturated in this assay.

Therefore we concluded that calcium chloride is the major causative agent of *N. benthamiana* aphid resistance. The fact that not only *N. benthamiana* but also *N. gossei* exhibit high calcium concentrations suggests that this mechanism of aphid resistance might be widespread.

Ma (1972) reported that calcium chloride was a feeding deterrent to *Pieris brassicae*. In our experiment, calcium chloride also showed significant antiaphid activity without supplying aphid food (Table 8). This result suggests that calcium chloride is toxic to aphids rather than a feeding deterrent.

Although there are a few reports on the effects that mineral salts borne by leaves have on aphids (Salas et al., 1990; Corcuera, 1993), this is the first report to our knowledge that shows calcium chloride exuded on the leaf surface is the major cause of the aphid resistance.

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RELATIVE IMPORTANCE OF SEMIOCHEMICALS FROM FIRST AND SECOND TROPHIC LEVELS IN HOST FORAGING BEHAVIOR OF *Aphidius ervi*

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Abstract—The responses of female *Aphidius ervi* to odors from a host food plant (*Vicia faba*), host aphids (*Acyrthosiphon pisum*), nonhost aphids (*Aphis fabae*), and aphid–plant complexes were investigated in a specially designed wind tunnel and a Y-tube olfactometer. In single-target (no-choice) and two-target (dual-choice) experiments, plant volatiles played a crucial role in the host foraging behavior of *A. ervi*. The odor from the *A. pisum*–plant complex elicited the strongest responses by *A. ervi* females, followed by the odor from plants previously damaged by the feeding of *A. pisum*. There was a significantly weaker response to odor from *A. pisum* in the absence of the plant and to undamaged plants. Similarly, mechanically damaged plants and plants infested with the nonhost aphid *A. fabae* did not elicit strong responses. A plant that had been damaged by *A. pisum* and subsequently washed with distilled water was as attractive as an unwashed, previously infested plant. *Aphidius ervi* probably overcomes the reliability–detectability problem by selectively responding to herbivore-induced, volatile, semiochemical cues emitted by the first trophic level and by distinguishing between the volatiles induced by host and nonhost aphids.

Key Words—*Aphidius ervi*, host location, olfaction, plant volatiles, aphid feeding, *Acyrthosiphon pisum*, *Aphis fabae*, wind tunnel, Y-tube olfactometer, tritrophic interactions.

INTRODUCTION

The host foraging behavior of female insect parasitoids has been divided into a series of steps that include habitat orientation, host community location, and

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host location (Vinson, 1985). Chemical cues play an important role in all of these stages, and such chemicals have been termed infochemicals (Vet and Dicke, 1992). The volatile chemical cues employed by parasitoids during host foraging may originate from the plant upon which the host is feeding, the host itself, other organisms associated with the host, or chemicals released as a result of interactions between the host and its food plant (Lewis and Martin, 1990). The utility of chemical information depends upon its reliability and its detectability (Vet and Dicke, 1992; Steinberg et al., 1993). Information originating directly from the host is the most reliable, but this is often difficult to detect, especially over longer distances, as the infochemicals are usually produced in minute amounts and must be detected against the background of odors in the environment (Vet and Dicke, 1992). On the other hand, plant volatiles are easily detected but have a low reliability as they do not guarantee the host's presence. According to Vet and Dicke (1992), such a dilemma can be overcome by the parasitoid in three ways: (1) by responding to conspicuous cues from stages of the host different from the one which is attacked; (2) by focusing on stimuli associated with interactions between the host and its food plant; and (3) by learning to link easy-to-detect cues with less detectable but reliable cues. There is now strong evidence that some parasitoids of lepidopteran pests, as well as certain predatory mites, use the second approach and respond to host herbivore-induced plant volatiles (Steinberg et al., 1993; Takabayashi et al., 1994; Tumlinson et al., 1993; Turlings et al., 1990, 1991; Vet and Dicke, 1992).

Much less is known about the role of chemical cues in the foraging behavior of aphid parasitoids (Hymenoptera, Braconidae, Aphidiinae) than is known about their role in the foraging behavior of parasitoids attacking lepidopteran hosts. Nevertheless, in several studies using Y-tube olfactometers, host plant volatiles were also important cues for foraging aphid parasitoids (Read et al., 1970; Schuster and Starks, 1974; Powell and Zhang, 1983; Wickremasinghe and van Emden, 1992). In contrast, females of the aphid parasitoid *Aphidius nigripes* did not respond to plant volatiles in the olfactometer but were strongly attracted by odors from host aphids (Bouchard and Cloutier, 1984). Furthermore, flight responses to odors from aphid hosts and uninfested or host-infested plants have been recorded in wind tunnels for *Diaeretiella rapae*, *Aphidius ervi*, and *Lysiphlebus testaceipes* (Scheehan and Shelton, 1989; Guerrieri et al., 1993; Grasswitz and Paine, 1993). Thus, different aphid parasitoid species appear to differ in their responses to chemical cues originating from different trophic levels in the system but, in the case of *A. ervi*, there is recent evidence that aphid-induced plant volatiles are important foraging cues (Guerrieri et al., 1993). In this study we extended these investigations of tritrophic influences on *A. ervi* foraging behavior, testing both nonhost aphid plant complexes and mechanically damaged plants in wind-tunnel bioassays and also developing bioassays involving a choice of two targets.

METHODS AND MATERIALS

A. ervi was reared on colonies of the pea aphid (*Acyrthosiphon pisum*) feeding on broad bean plants (*Vicia faba*). The black bean aphid (*Aphis fabae*) was also reared on *V. faba*. Aphid and parasitoid cultures were maintained in separate environmental chambers operating at 18 ± 1°C, 75 ± 5% relative humidity, and a 18L:6D photoperiod. Prior to their use in experiments, adult female wasps were mated within 24 hr of emergence and kept in cages with access to honey solution (50%) under the same environmental conditions as described above. All parasitoids used in the tests were 2 days old and naive, having had no exposure to either aphids or plants since emergence, and each individual was used only once. Most of the experiments were done using a laboratory culture that was established in 1992 from approximately 20 females reared from *A. pisum* collected in a lucerne crop on Rothamsted farm (British strain). For comparison, some experiments were also done using a culture established in 1992 from approximately 30 females reared from *A. pisum* in lucerne crops in Bulgaria (Bulgarian strain). All experiments were done 8–12 hr after the start of the 18-hr light period of the photoperiodic regime under which the parasitoids were reared.

Wind-Tunnel Experiments

The flight responses of *A. ervi* females to a range of olfactory cues were investigated using a wind tunnel that was specially designed for studying parasitoid flight behavior. This consisted of a rectangular Plexiglas chamber (90 × 30 × 30 cm) through which air was pulled by an electronically operated fan, controlled by a variable voltage regulator. Air entering the flight chamber was purified by passing through a charcoal and glass wool filter, and a laminar flow was obtained by means of a perforated mesh panel that reduced the eddy size and created a uniform airstream. Mesh panels were present at both ends of the tunnel to increase the uniformity of the airstream, essential for obtaining well-defined odor plumes. Titanium chloride and smoke from a candle wick were used to test plume structure with various targets present in the wind tunnel. The wind speed in all experiments was adjusted to 23 ± 2 cm/sec. Exhaust air was vented outside the room so as to avoid contamination of the room with test odors. Light intensity in the wind tunnel was 3600 lux at the insect-releasing site and the lighting was supplied by three U-shaped incandescent lights suspended above the tunnel. Tissue paper was placed between the lights and the tunnel roof to diffuse the light and thus prevent parasitoids from flying directly upwards after release. A black and white striped pattern was placed beneath the tunnel floor to aid visual orientation by flying parasitoids. All plants used in the

experiments were 10 cm high broad bean seedlings (cv. Sutton) with two or three pairs of leaves.

No-Choice Experiments. Single-target (no-choice) experiments were done using the following treatments: (1) host aphids (100–150), *A. pisum*, presented in a glass tube that was open at both ends and placed horizontally in the air-stream, nylon mesh over the ends of the tube prevented aphid escapes. (2) an uninfested plant, (3) a plant infested with 100–150 *A. pisum* 24 hr before testing (plant–host complex), (4) a plant that had previously been infested with 100–150 *A. pisum* but from which the aphids had been removed immediately before use in the bioassay (host damaged plant), (5) an uninfested plant that had been mechanically damaged using a pin to pierce the leaves, 100 pin pricks were administered to the plant immediately before the experiment, and (6) a plant infested with 100–150 *Aphis fabae*, an aphid species that is not a host for *A. ervi*.

Between 50 and 90 parasitoid females were tested individually by releasing them in the odor plume 20 cm downwind from the target, the release distance used by Guerrieri et al. (1993). They were released from a glass cylinder (4.5 cm diameter, 7.3 cm long), open at both ends, which was placed horizontally 10 cm above the tunnel floor and positioned so that the odor plume could pass through. The number of parasitoids taking off from the release site, the number performing direct flights oriented towards the target, and the number landing on the target were recorded. Each female was allowed a maximum of 5 min to respond and given only one opportunity to fly. The mean percentage response per day was calculated for each of the treatments (treatments were randomized so as to eliminate any time variation). Daily mean values were then averaged to give the overall response $\pm$ SE. The proportions responding (showing oriented flight and landing on the target) were subjected to logit transformation to normalize the data and then analyzed using a two-way analysis of variance to test the effect of strain and treatment on the response. Tukey multiple comparison tests were used to compare individual treatment means.

For all treatments, except plants infested with *A. fabae*, two strains of *A. ervi*, one originating in the UK and the other in Bulgaria, were tested and compared. Because both the British and Bulgarian strains of *A. ervi* showed the same patterns of responses in the single-target experiments, only the British strain, which was more readily available, was used in all other experiments.

Screen Experiments. To reduce the possible influence of visual cues on the flight responses, further tests were done in which a fine mesh screen was placed in front of the target, either uninfested plants or the plant–host complex. Responses to screened targets were compared with those to unscreened targets. Three treatments were used: (1) an uninfested *V. faba* plant, (2) a screened, uninfested plant, and (3) a screened plant that was infested with 100–150 *A. pisum*, 24 hr before the experiment.

The experiment was repeated on three separate days and a minimum of 12 parasitoids were tested for each treatment on each day. The proportions responding (showing oriented flight and landing on the target) were subjected to logic transformation to normalize the data and then analyzed using a one-way analysis of variance. Tukey multiple comparison tests were used to compare individual treatment means.

Dual-Choice Experiments. The dual-choice experiments were undertaken to reduce the problem of day-to-day variation in parasitoid responsiveness, without the need for complex randomizations or trying to complete sufficient replications of all no-choice treatments in any one day. Dual-target (choice) experiments were done using the following target combinations: (1) an uninfested plant vs. the plant–host complex, (2) an uninfested plant vs. a host-damaged plant, (3) the plant–host complex vs. a plant infested with the nonhost *A. fabae*, (4) an uninfested plant vs. a plant infested with *A. fabae*, (5) an uninfested plant vs. a mechanically damaged plant, (6) an uninfested plant vs. host aphids (*A. pisum*), (7) the plant–host complex vs. a host-damaged plant, and (8) a host-damaged plant vs. a host-damaged plant that had been washed to remove water-soluble compounds associated with aphid honeydew and exuviae.

All the plant–aphid complexes consisted of a *V. faba* plant that had been infested with 100–150 aphids (either *A. pisum* or *A. fabae*) 24 hr before the experiment. The targets were placed 10 cm apart, and smoke tests showed that they produced separate plumes that began to merge approx. 45 cm downwind. Therefore, the parasitoid release tubes were sited a little over 45 cm downwind from the targets. The number of parasitoids taking off and the number landing on one of the targets were recorded. Each parasitoid was allowed 5 min to respond and between 88 and 110 individuals were used for each experiment. A χ^2 test was used to test whether there was a significant preference between the targets. Only those parasitoids that landed on one of the targets were included in the analysis.

Y-Tube Olfactometer Experiments

A Y-tube olfactometer was also used to investigate the responses of *A. ervi* females when presented with a choice of odors in the absence of any visual cues. A pump was used to pull air through activated charcoal and a molecular sieve so that it was purified of any contaminating odors before passing over the target and entering the Y-tube. The airflow through each of the olfactometer arms was maintained at 800 ml/min. In the experiments parasitoids were given a choice between an undamaged plant and either *A. pisum* aphids or the plant–host complex. Fifty female parasitoids were tested, individually, in each experiment.

The first choice of each parasitoid was noted as well as its final choice after 5 min. If during these 5 min it reached the end of the arm of its choice, this was regarded as a final choice. A χ^2 test was used to determine significant differences between numbers of parasitoids choosing different odor treatments or arms of the olfactometer.

RESULTS

Wind-Tunnel Experiments

No-Choice Experiments. ANOVA analysis showed that both parasitoid strain ($P < 0.05$) and treatment ($P < 0.001$) significantly affected the parasitoid response, but there was no interaction ($P = 0.17$). A greater proportion ($P < 0.05$) of the Bulgarian strain landed on the targets compared with the British strain (Figure 1). For both strains the plant–host complex and the host-damaged plant were equally attractive, and both these targets were significantly more attractive than either the undamaged plant and host aphids ($P < 0.05$). Mechanical damage did not significantly increase the attractiveness of a plant undamaged by aphids, although both undamaged and mechanically damaged plants were more attractive than aphid hosts that had been removed from the plant (Figure 1). The presence of the nonhost aphid *A. fabae* on plants did not significantly increase parasitoid responses, compared with those to undamaged plants, although this treatment was only tested with the British strain of *A. ervi* (Figure 1). These results were unaltered when those parasitoids that exhibited oriented flight but failed to land on the target were included in the analysis.

Screen Experiments. A significantly greater percentage of parasitoids ($P < 0.05$) landed on a screened plant–host complex than on either a screened or unscreened undamaged plant (Figure 2). The responses to screened and unscreened undamaged plants were not statistically different (Figure 2).

Dual-Choice Experiments. In all choice experiments, 88% or more of the parasitoids took off from the release tube and oriented upwind, but the number is not presented as we were interested in the final choice between the targets. There were no significant differences in the percentage landing on the two targets in choices between: the plant–host complex and a host-damaged plant (22.7%:17.0%); a host-damaged plant and a washed host-damaged plant (22.3%:20.2%); an undamaged plant and a plant infested with *A. fabae* (11.8%:17.6%); and an undamaged plant and a mechanically damaged plant (11.6%:8.4%) (Figure 3).

Significantly more parasitoids landed on the plant host complex than on an undamaged plant (35.5%:10.7%; $P < 0.05$) and significantly more landed on a host-damaged plant than on an undamaged plant (20.0%:3.8%; $P < 0.05$) (Figure 3). In the choice test between a plant infested with the host *A. pisum*

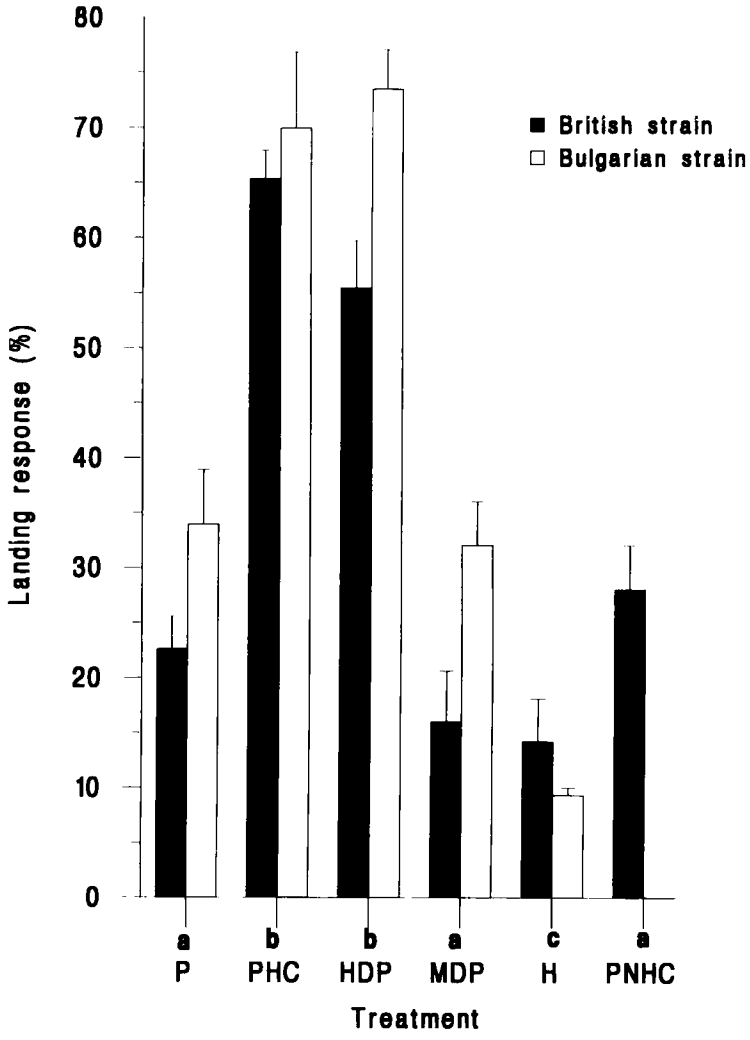

FIG. 1. Percentage of British and Bulgarian strain *Aphidius ervi* females landing on targets in no-choice wind-tunnel experiments. P: undamaged *Vicia faba* plant; PHC: plant-host (*Acyrthosiphon pisum*) complex; HDP: host-damaged plant (aphids removed); MDP: mechanically damaged plant; H: host (*A. pisum*); PNHC: plant-nonhost (*Aphis fabae*) complex. Two-week-old *V. faba* seedlings were used in the experiments. Analysis of variance was performed on the data after a logit transformation. Treatments with different letters beneath were significantly different ($P < 0.05$) based on Tukey multiple comparison tests.

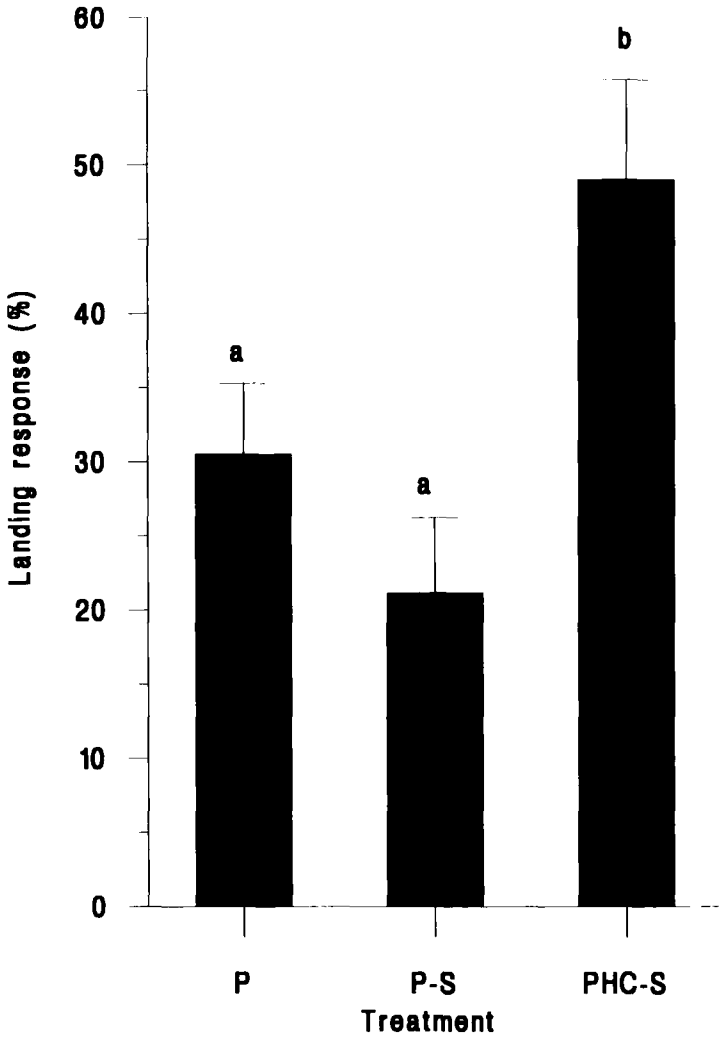

FIG. 2. Percentage of *Aphidius ervi* females landing on undamaged *Vicia faba* plants (P) and on a screen placed in front of either an undamaged plant (P-S) or a plant–host (*Acyrthosiphon pisum* complex, (PHC-S) in no-choice wind-tunnel experiments. Analysis of variance was performed on the data after logit transformation. Treatments with different letters were significantly different ($P < 0.05$) based on Tukey multiple comparison tests.

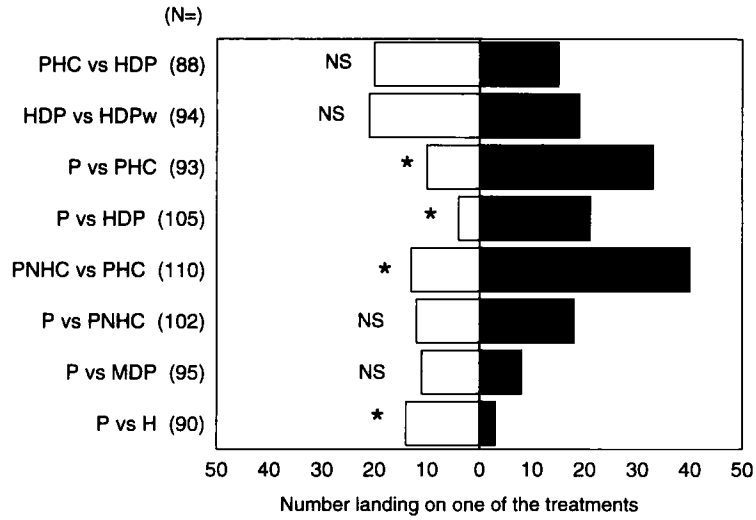

FIG. 3. Percentage of *Aphidius ervi* females landing on each target in dual-choice wind-tunnel experiments. P: undamaged *Vicia faba* plant; PHC: plant–host (*Acyrthosiphon pisum*) complex; HDP: host-damaged plant (aphids removed); HDPw: host-damaged plant (aphids removed) washed with distilled water; MDP: mechanically damaged plant; H: host (*Acyrthosiphon pisum*); PNHC: plant–nonhost (*Aphis fabae*) complex. Two-week-old *V. faba* seedlings were used in the experiments. (*Significant difference between targets, χ^2 test, $P < 0.05$).

and a plant infested with the nonhost aphid *A. fabae*, significantly more parasitoids landed on the former (36.4%:11.8%; $P < 0.05$). Finally, significantly more parasitoids landed on an undamaged plant than on an open-ended tube containing host aphids (15.6%:3.3%; $P < 0.05$).

Y-Tube Olfactometer Experiments

In both experiments [undamaged plant (P) vs. the plant–host complex (PHC) and undamaged plant vs. host (H) aphids] there were no significant differences between the numbers of parasitoids entering each of the two olfactometer arms as a first choice (22 P vs. 26 PHC, $P > 0.05$; 28 P vs. 21 H, $P > 0.05$) (Figure 4). However, significantly more ($P < 0.05$) made a final choice of the plant–host complex (32) than chose the undamaged plant (14) and significantly more ($P < 0.05$) made a final choice of the undamaged plant (31) than chose the host aphids, *A. pisum* (14). Of the 50 parasitoids used in each experiment only two in the first experiment and one in the second failed to enter either

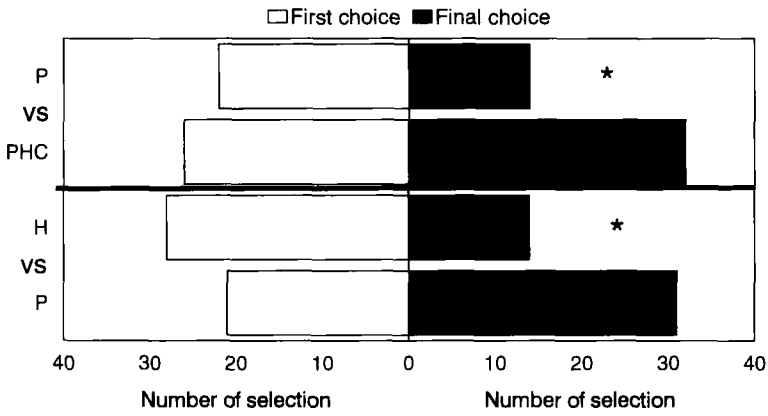

FIG. 4. Responses of *Aphidius ervi* females to undamaged plant *Vicia faba* (P) vs. host aphids (*Acyrthosiphon pisum*) (H) and plant–host (*A. pisum*) complex (PHC) vs. undamaged plant (P) in a Y-tube olfactometer. (*Significant difference between arms in final choices only, χ^2 test, $P < 0.05$).

olfactometer arm, while two in the first and four in the second failed to make a definite final choice after 5 min. Most of the parasitoids either remained in the first arm they entered or changed arms only once.

DISCUSSION

Our results confirm the main conclusions of the study by Guerrieri et al. (1993), that both the *Vicia faba/Acyrthosiphon pisum* plant–host complex and plants damaged by *A. pisum* (but from which the aphids had been removed) are more attractive odor sources than either undamaged plants or aphids alone. The response of parasitoids to odors in the Y-tube olfactometer and the flight responses to screened targets in the wind tunnel indicate that visual cues are not essential, at least in the long-range stages of *A. ervi* foraging behavior. As many parasitoids (almost 50%) orient upwind to odors from the plant–host complex placed behind a screen, it proves that odor alone can elicit oriented upwind flight. Subsequent experiments with the wind tunnel have shown that parasitoids will fly upwind to filter paper targets treated with chemical extracts isolated from the plant–host complex (Du et al., unpublished), emphasizing that the visual cue provided by the plant is not essential for a response. However, visual cues are known to be important in later stages of the host location process of some parasitoids (Michaud and Mackauer, 1995; Wäckers, 1994).

The weak responses observed to undamaged plants and to host aphids in the absence of the plant suggest that chemicals emitted from these sources are not efficient cues for foraging *A. ervi* during host habitat and host location. In fact, the weakness of the flight response to host aphids in a glass tube is emphasized by the fact that some landing (8%) occurred on an empty glass tube. Thus, although aphid-derived volatile cues are very reliable, because they guarantee the presence of the host, they appear to be much less important as *A. ervi* flight orientation cues than are volatiles from the plant–host complex, presumably because they are relatively difficult to detect. In contrast, plant odors are much more detectable but, unless they are linked directly to the presence of the parasitoids' host, are not very reliable beyond the location of appropriate habitats in which to forage. *A. ervi* has overcome this problem by evolving strong responses to plant odors directly associated with host damage and can distinguish from a distance between a plant infested with or recently damaged by host aphids and an undamaged plant. The release of herbivore-induced plant volatiles that act as reliable and detectable host-location cues for parasitoids and predators has previously been demonstrated for several other tritrophic systems (Dicke et al., 1990; Turlings et al., 1990; Whitman and Eller, 1990). The fact that *A. ervi* responded just as strongly to host-damaged plants whether or not the aphids were still present on the plant suggests that aphid-induced volatiles are the main stimuli. This is reinforced by the continued strong response by parasitoids to damaged plants from which aphid honeydew and exuviae have been removed by washing with water. It is still possible, of course, that some aphid-derived, non-water-soluble chemicals remained on the leaf surfaces.

The reliability of the chemical cues produced by aphid-damaged plants would be greatly reduced if the same range of plant volatiles was emitted following all forms of plant damage. Mechanical damage to plants is known to increase their attractiveness to parasitoids in some tritrophic systems (Elzen et al., 1993; Eller et al., 1988a,b; Ding et al., 1989; Turlings et al., 1990; McAuslane et al., 1991; Steinberg et al., 1993). However, mechanical damage to the leaves of *V. faba* plants did not increase their attractiveness to *A. ervi* in our wind-tunnel trials, but this result needs to be treated with caution because the plants were damaged immediately before they were used as targets. Plants may have been tested too quickly for the completion of changes in volatile emissions induced by the damage. There is evidence from other studies that the emission of specific plant volatiles following mechanical damage is intermittent and that the induced changes in emissions can wane very quickly unless the damage is repeated at regular intervals (Tumlinson et al., 1992; Steinberg et al., 1993).

Mechanical damage to plants results in an increased emission of C_6 alcohols, aldehydes, and their esters, the so-called green leaf volatiles (Mattiacci et

al., 1994). Although several parasitoid species are known to be attracted to these green leaf volatiles (Whitman and Eller, 1990), their ratio can be important in determining an insect's perception of the plant (Visser et al., 1979; Visser, 1986). Changes in volatile ratios due to mechanical damage have been reported to confuse the perception of aphids to host plant odors (Nottingham et al., 1991) and similar confusion may also have occurred with *A. ervi*, causing its lack of response to the mechanically damaged target. Further work on this aspect is obviously needed. Nevertheless, it has been shown that some natural enemies do discriminate between the volatiles of a mechanically damaged plant and those of an herbivore-damaged plant (Vet and Dicke, 1992; Turlings et al., 1990; Dicke, 1994).

The specificity, and therefore reliability, of the semiochemical cues emitted by the plant–host complex is emphasized by the fact that *A. ervi* showed the same weak response to plants infested by the nonhost aphid *A. fabae* as it did to undamaged plants. Thus *A. ervi* appears to have overcome the reliability–detectability problem (Vet and Dicke, 1992), by responding to olfactory cues produced by the plant when it is damaged by an appropriate host species. The dual-choice experiments demonstrated that *A. ervi* females could distinguish between plants infested with each of the two aphid species tested, when equal numbers of aphids had been feeding on the plants for the same length of time. However, it is possible that different aphid species stimulate the production of host-induced volatiles by the plant at different rates, which could account for parasitoid choice in the wind-tunnel tests. This is currently being investigated by generating dose–response curves for the two aphid species. Nevertheless, different aphid species may induce different changes in plant volatile emissions, and comparisons of the feeding biology of these two aphids may provide clues to the physiological and biological processes involved in herbivore-induced volatile emission. Air entrainment samples of uninfested bean plants and plants infested with *A. pisum* or *A. fabae* had significantly different chemical compositions (Du et al., unpublished). Previous studies of other tritrophic systems have also produced evidence that herbivore-induced plant synomones may be specific for the herbivore species involved (Dicke, 1988, 1994; Turlings et al., 1990; Takabayashi et al., 1991). In the single-target wind-tunnel experiments it was not possible to complete tests of all targets within one day, and it was observed that the general responsiveness of the parasitoids could vary from day to day. Such variation has been noted in other wind-tunnel studies with parasitoids and may be caused by changes in barometric pressure and, in particular, the direction of those changes during the course of an experiment (Steinberg et al., 1992). However, the results from the choice experiments confirmed the hierarchy of responses deduced from the single-target experiments, even though the target distance in the dual-choice experiments was greater (45 cm) than in the no choice experiments (20 cm). The overall percentage of parasitoids landing

on the plant–host complex and host-damaged plant targets was lower in the choice experiments (Figure 3) than when these targets were presented alone (Figure 1), which may have been partly due to the increased flight distances.

Although the Y-tube olfactometer detects short-range responses to volatile cues by walking parasitoids, whereas the wind tunnel detects long-range flight responses, the results suggest that the same plant-associated semiochemical cues are playing a role in both situations. Previous studies had shown that *A. ervi* females responded to both *A. pisum* and *Vicia faba* leaves in a Y-tube olfactometer when these were tested separately (Powell and Zhang, 1983). The parasitoids were presented with a choice in the present study that demonstrated a preference for the undamaged plant over *A. pisum*. The aphid-derived semiochemical cues may operate most efficiently at close range during the later stages of host location, when visual cues also come into play.

A legitimate criticism of many Y-tube studies is a failure to account for wrong initial decisions made by the insect while searching within the apparatus. This was overcome by recording the final choice of olfactometer arm after a set time period. Many of the parasitoids that made a "wrong" first choice either reversed their decision or failed to make a definite final choice of olfactometer arm. Thus, if the final choice is considered, this type of olfactometer is useful as a fast and efficient initial screen in foraging behaviour studies.

In the tritrophic system involving *V. faba*, *A. pisum*, and *A. ervi*, naive parasitoids overcome the reliability–detectability problem by utilizing cues coming from the plant when the appropriate host aphid has fed upon the plant. Recent work indicates that experienced *A. ervi* females also use the third solution proposed by Vet and Dicke (1992), associative learning, to overcome this problem (Du et al., in prepration). Our data suggest that an active series of physiological and biochemical processes occur within the plant as a result of aphid feeding, leading to quantitative and/or qualitative changes in the volatiles emitted, and that the parasitoid can distinguish between plants infested with host and nonhost aphid species. We are currently investigating how many aphids and for how long (aphid hours) are needed to induce these changes, how long the effects last once the aphids have been removed, and whether there is a systemic release of herbivore-induced plant signals from uninfested parts of the plant.

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ELECTROANTENNOGRAM ACTIVITY FROM ANTENNAE OF *Ceratitis capitata* (WIED.) TO FRESH ORANGE AIRBORNE VOLATILES

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Abstract—Twenty-six compounds have been identified from fresh orange airborne volatiles, the most abundant being limonene and alloaromadendrene. Ethyl- and butylhexanoates were found in middle concentrations, whereas the other compounds occur at low levels. Electroantennograms recorded from *Ceratitis capitata* Wied. (males and females) revealed limonene, butyl hexanoate, dihydrocarvone, and limonene oxide to be most active. In the case of dihydrocarvone, 4-ethyl acetophenone, and carvone, female antennae give greater responses than antennae of males. Limonene oxide was more active for males than females. Total airborne volatiles recovered from oranges elicited high EAGs, with females being more sensitive than males.

Key Words—Oranges, fruit volatiles, odor, *Ceratitis capitata*, Mediterranean fruit fly, Diptera, Tephritidae, olfaction, electroantennogram, electrophysiology.

INTRODUCTION

Ceratitis capitata (Wied.) is a highly polyphagous species capable of adapting itself to a great variety of fruits. In Florida (USA) up to 175 potential host plant species are described including apricots, peaches, nectarines, oranges, cherries, and plums. Females respond to host-plant stimuli such as color, shape, size,

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and volatile substances of the fruit, and oviposit by sticking the tip of the ovipositor into the epidermis and laying eggs in small two- to eight-unit packs (Hippe, 1987; Levinson et al., 1987). When the larvae hatch they feed by tunneling through these fruits.

Trimedlure, a synthetic attractant, attracts only males and mimics the sexual pheromone or the lek-forming pheromone (Prokopy and Roitberg, 1984; Shelly et al., 1993). In order to develop new less-polluting detection and control methods, it may be useful to find female-attracting compounds. These could give rise to a great number of applications, since they could be combined with pesticides, sterilizing agents, and entomopathogenic microorganisms in strategically situated traps.

Several papers have been published on plant components, extracts, and essential oils that attract *C. capitata* females and males (Teranishi et al., 1987; Warthen and McInnis, 1989). From the essential oil of *Angelica arcangelica* α-copaene was isolated; it is more attractive to males than trimedlure, although females are not attracted. α-Copaene was later isolated from other essential oils, including that of orange (Fornasiero et al., 1969; Jacobson et al., 1984). Guerin et al. (1983) and Light et al. (1988) found that the antennae of *C. capitata* are more sensitive to compounds with six carbon atoms than to other chain lengths. Dickens et al. (1990) observed an increase in the number of females attracted to traps when green leaf volatiles [especially (*E*)-2-hexenal] were added to the volatiles released by males.

Within this scope, we have identified volatile compounds emitted by oranges (OAV) and studied their EAG activity on antennae of males and females of *C. capitata*.

METHODS AND MATERIALS

Oranges (navel variety) were collected in Buñol (Valencia, Spain) during March (1991–1992). Fruits were introduced into a sealed glass bottle through which a clean airflow (60 ml/min) was passed. The air was cleaned by passing successively through filters of 20% potassium permanganate, charcoal, and Porapak Q. The airflow carried the orange airborne volatiles (OAV) into a stainless steel column (15 × 1 cm) packed with 10 mg porapak Q (Jang et al., 1989). Approximately 10 fruits were placed into the bottle and changed every four days up to a period of 20 days. Volatiles trapped on Porapak Q were extracted by a rinse with 20 ml of dichloromethane followed by pentane in a Soxhlet extractor for 12 hr.

OAV were analyzed by GLC using a Perkin-Elmer Sigma 3B gas chromatograph with a flame ionization detector and Hewlett Packard HP3396A recorder. The analytical column was HP Ultra 1 (methyl silicone gum; 25 m ×

0.2 mm × 0.33-μm film thickness). Chromatography conditions were: initial temperature 50°C; rising 4°C/min to 180°C and then 10°C/min to 280°C; N_2 was the carrier gas at 1.8 ml/min.

In order to identify the minor components, the total extract was fractionated with the same chromatograph to which a 2:8 splitter was coupled at the end of the column to collect fractions. For this purpose an HP-1 column (methyl silicone gum 5 m × 0.53 mm × 2.65-μm film thickness) was used. Conditions were: initial temperature 50°C, 5 min; rising 3°C/min to 120°C and then 10°C/min to 280°C; N_2 was the carrier gas at 6 ml/min. The different fractions were collected in stainless steel capillary tubes having a U-shaped loop immersed in acetone–Dry Ice (25:75) at −70°C, and an end branch placed into a glass tube containing 0.5 ml dichloromethane kept at −70°C. Capillaries were washed with dichloromethane.

Fractions were analyzed by GLC-MS using a Hewlett Packard 5988A quadrupole with electronic impact at 70 eV. The analytical column was HP Ultra 2 (5% phenyl methyl silicon; 25 m × 0.2 mm × 0.33-μm film thickness). Chromatography conditions were: initial temperature 50°C; rising 4°C/min to 180°C and then 10°C/min to 280°C; He was the carrier gas at 1.8 ml/min).

Identification was also verified through calculation of Kovats index numbers and standards provided by Aldrich Chemical Company Inc. (Steinheim, Germany), Sigma Products (Deisenhofen, Germany), Fluka Chemie AG (Buchs, Switzerland), Alfa Products (Karlsruhe, Germany), Extrasynthese (Genay, France), and Merck-Schuchhardt (Darmstadt, Germany); or synthetized in our laboratory.

Activity Assays in EAG. C. capitata was reared at 26 ± 1°C, 70 ± 5% relative humidity and 16L:8D photoperiod. Adults were separated by sex and kept on a diet of sugar, hydrolyzed proteins, and water. Virgin males and females were used five to six days after emergence.

Compounds at six different doses between 10^{-2} and 10^3 μg dissolved in 10 μl of dichloromethane were impregnated on glass fiber filter paper (1 cm^2). The solvent was evaporated, and the paper was introduced into a Pasteur pipet. A flow of 300 ml/min air was passed for 1 sec through the Pasteur pipet and mixed with a 1 liter/min humid airstream at a point 5 cm from the insect's antenna. Dichloromethane (10 μl) was used as a control and 100 μg of hexanol as standard. There were no significant differences between male and female responses to the standard signal (7.8 ± 0.8 mV). The response of each substance was corrected with those of the control (0.5 ± 0.02 mV), and they were given with respect to the standard according to the following formula: {[mV(test) − mV(control)] × 100}/[mV(hexanol) − mV(control)]. Each assay (one substance × six doses) was repeated with five different males and females. Air was passed during a 2-min interval between tests (Light et al., 1988). A silver recording electrode made contact with the third antennal segment, whereas the

silver indifferent electrode was immersed in saline Ringer's solution. The measuring system consists of two nonamplifying buffers, each of them connected to an electrode for signal conditioning, with a low impedance output. Both output systems were connected to an amplifying module; a high-precision DC level fixer and a low-pass filter were used to deal with the contact potentials that were amplified. The signal was recorded by means of a Tektronic TDS 310 oscilloscope coupled with an IBM PC-AT and a Hewlett Packard LaserJet IIP printer.

Statistical data management was carried out by a mixed analysis of variance (ANOVA), which takes into account not only the structure of the experiment (mixture of crossed and hierarchical factors) but also the nature of factors (combination of fixed and random effects) (Kendall and Stuart, 1976; Dixon, 1992). Apart from simple effects, the statistical model evaluates the following interactions: substance–sex, substance–concentration, concentration–sex, and substance–concentration–sex.

RESULTS

Composition of Fresh Orange Airborne Volatiles. Orange volatiles collected on Porapak Q were fractioned into four parts by using semipreparative GLC with a splitter. Figure 1 shows the analytical chromatograms of the four fractions. The compounds were identified by GLC-MS, Kovats index, and in some cases by comparison with authentic standards (Table 1). The most abundant substances were limonene and alloaromadendrene. 1,2-Diethylbenzene, 1,3-diethylbenzene, *n*-butyl *n*-hexanoate, and *n*-hexyl *n*-hexanoate were found at intermediate levels, whereas very small amounts of the other compounds were detected.

Activity Assays. Figure 2 shows EAG responses for 13 identified products at six concentrations tested with both females and males. Table 2 summarizes the ANOVA results and shows a statistical significance for most terms of the model. The significance of the interactions in the product factor also shows that the differences in the EAG responses between compounds depend not only on their load on filter paper but also on the sex of the fly.

The response was greater at the highest doses along the range tested, except for 4-ethyl acetophenone, which yielded a maximum response at 100 μg from female antennae. For other substances, the antennae become saturated at low doses, for example, at 10 μg in the case of carveol and nootkatone (both sexes); at 10 μg and 100 μg for carvone and 4-ethyl acetophenone, respectively (males); and at 100 μg for dihydrocarvone (females). P $\times$ C interaction indicated a difference in the effect of concentration (within the range tested) depending on the product used. The increase in the response with increased concentration was

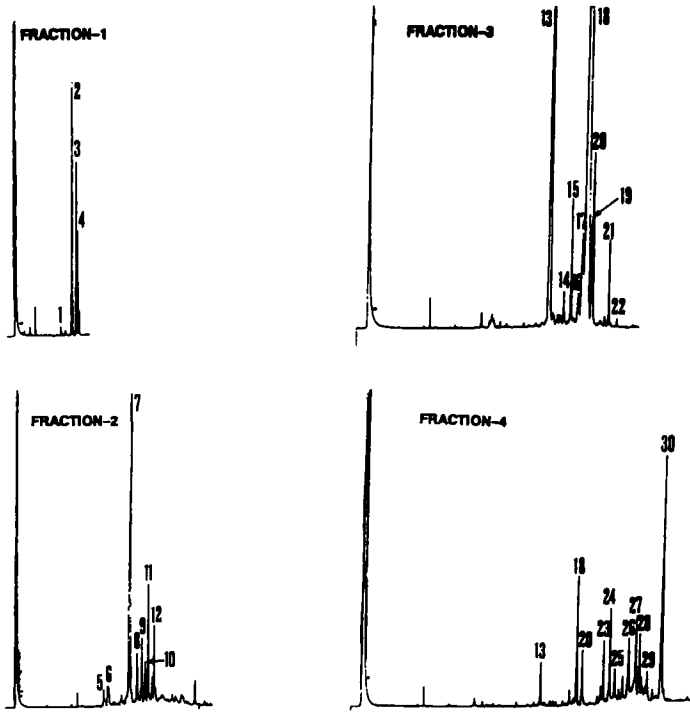

FIG. 1. GC of fractions of the fresh orange airborne volatiles in HP Ultra 1 column, 25 m × 0.2 mm × 0.33-μm film thickness (initial temperature 50°C; rising 4°C/min to 180°C and then 10°C/min to 280°C, N_2 was the carrier gas at 1.8 ml/min). Flame ionization detector. Fractions have been obtained by semipreparative GC with HP1 column, 5 m × 0.53 mm × 2.65-μm film thickness (initial temperature 50°C, 5 min, rising 3°C/min to 120°C and 10°C/min to 280°C, N_2 was the carrier gas at 6 ml/min), the first fraction was collected up to min 3, the second from 3 to 12 min; the third from 12 to 20 min, and the fourth from 20 min to 35 min. 1: 1-Isopropyl-4-methylbenzene, 2: limonene, 3: 1,2-diethylbenzene, 4: 1,3-diethylbenzene, 5: limonene oxide, 6: menta-2,8-dien-1-ol, 7: butyl hexanoate, 8: dihydrocarvone, 9: carveol, 10: carvone, 11: 1-Ethyl acetophenone, 13: hexyl hexanoate, 14: sesquiterpene, 15: aromadendrene, 16: β-copaene, 17: clovene, 18: alloaromadendrene, 19: γ-bisabolene, 20: δ-cadinene, 21: cascarilladiene, 22: caryophyllene oxide, 25: globulol, 26: spathulenol, 27: 2,6-di-*t*-butyl-1,4-benzoquinone, 29: 1(10)-aristolen-2-one, 30: nootkatone.

TABLE 1. COMPOUNDS IDENTIFIED IN FRESH ORANGES AIRBORNE VOLATILES[a]

	Substance	RT (min)	Area (%)	KI	
				Lit.	Exp.
1	1-Isopropyl-4-methylbenzene[1]	5.4	0.6	998[I]	980
2	Limonene[1][a]	6.0	22.0	1020[II]	1010
3	1,2-Dimethylbenzene[1][b]	6.1	14.9		1017
4	1,3-Diethylbenzene[1][a]	6.2	7.0		1023
5	Limonene oxide[1][c]	8.6	1.4	1119[II]	1101
6	Menta-2,8-dien-1-ol[1]	9.0	1.1	1120[II]	1114
7	Butyl hexanoate[2][d]	11.2	8.9	1173[III]	1179
8	Dihydrocarvone[2][c]	11.8	0.5	1187[IV]	1197
9	Carveol[1][c]	12.3	0.7	1204[IV]	1211
10	Carbone[1][c]	12.6	tr	1223[II]	1222
11	Ethyl acetophenone[1][a]	12.9	1.3		1232
13	Hexyl hexanoate[1][d]	17.7	7.2	1371[II]	1375
14	Sesquiterpene ($C_{15}H_{24}$)	19.1	tr		1415
15	Aromadendrene[2]	19.8	0.9		1438
16	β-Copaene[2]	20.5	tr	1460[II]	1460
17	Clovene[3]	20.8	1.4		1472
18	Alloaromadendrene[2][c]	21.4	21.5	1455[I]	1490
19	γ-Bisabolene[1]	21.7	1.3	1500[II]	1499
20	δ-Cadinene[1]	21.9	1.0	1513[I]	1504
21	Cascarilladiene[1]	23.5	tr		1559
22	Caryophyllene oxide[4][a]	24.1	tr	1567[I]	1580
25	Globulol[5]	24.6	tr		1596
26	Spathulenol[1]	26.0	tr		1644
27	2,6,-Di-*t*-butyl-1,4-benzoquinone[1]	26.7	0.6		1668
29	1(10)-Aristolene-2-one[1]	27.8	tr		1707
30	Nootkatone[2][c]	29.3	2.0	1775[V]	1759

[a]RT: retentiontime on an Ultra 1 analytical column, 25 m × 0.2 mm × 0.33-μm film thickness (initial temperature 50°C, rising 4°C/min to 180°C and then 10°C/min to 280°C: N_2 was the carrier gas at 1.8 ml/min). Flame ionization detector. tr: trace (area less than 0.5%). Lit.: literature, Exp.: experimental. KI: Kovats index: [1]Binder *et al.* (1990), [II]McLeod *et al.* (1988), [III]Flath *et al.* (1990), [IV]Tang *et al.* (1990), [V]Tamura *et al.* (1993). CGL-MS: [1]Anonymous (1991), [2]Stenhagen *et al.* (1974), [3]Doyle *et al.* (1965), [4]De Pascual *et al.* (1983), [5]Pakrashi *et al.* (1980). Confirmed with a authentic standard: [a]Aldrich Chemical Company, Inc, Germany; [b]Merck-Schuchhardt, Germany; [c]Janssen Chimica, Belgium; [d]prepared in our laboratory; [e]Extrasynthese, France.

greatest for limonene, limonene oxide, dihydrocarvone, and caryophyllene oxide, and smallest for 1,3-diethylbenzene and nootkatone. The significance in P × S interaction is due to the high response of limonene oxide in males and dihydrocarvone in females.

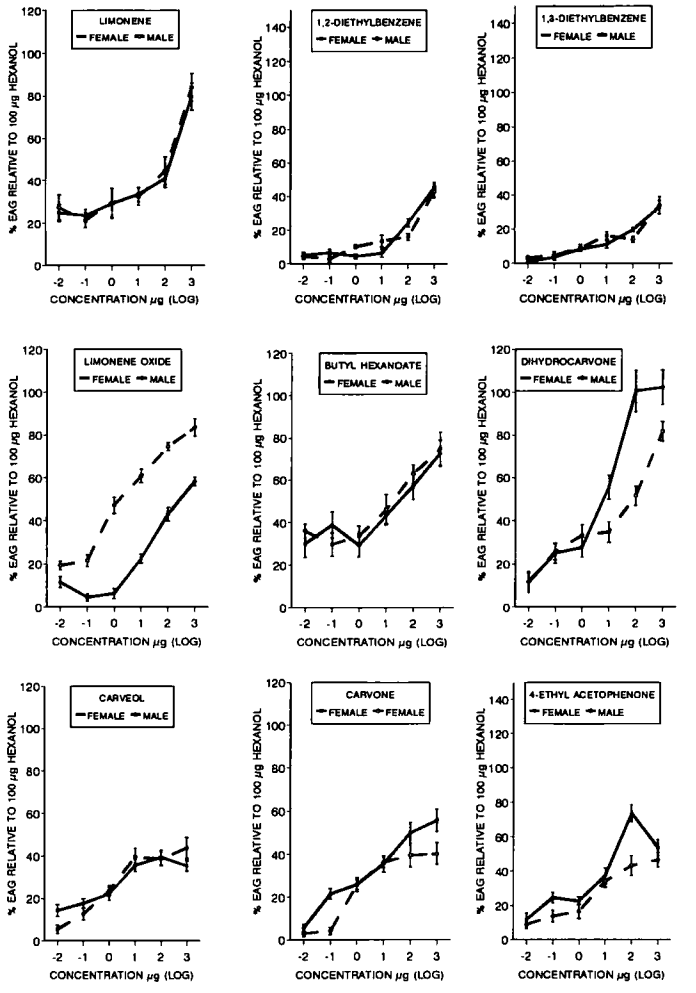

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FIG. 2. Dose-response curves of EAGs for *C. capitata* females and males at serial dilutions (from 10^{-2} to 10^{3} µg) of substances identified in fresh orange airborne volatiles. Vertical lines show the ± standard error (N = 5). A 100% response approximately corresponds to 8 mV.

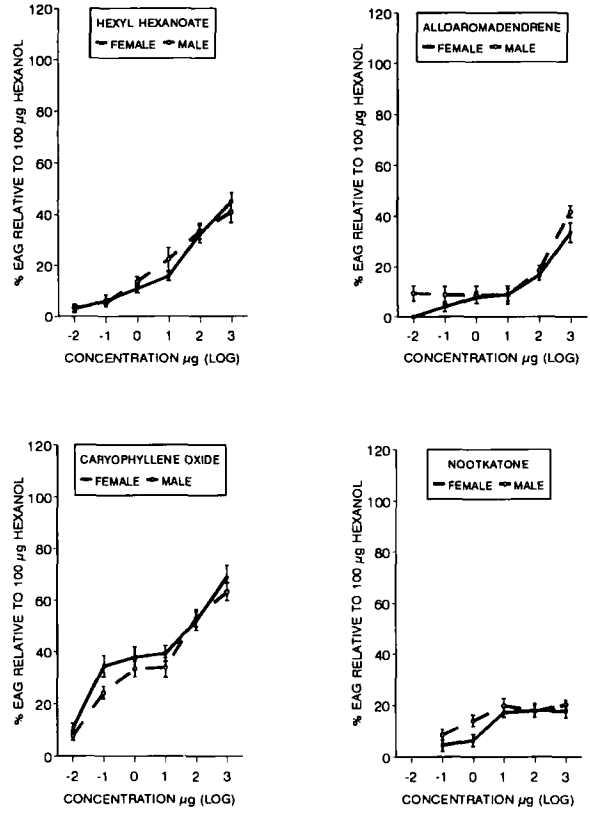

FIG. 2. Continued

Figure 3 shows the responses to each substance at their optimum doses and that of the total extract of OAV at the 1000-µg dose. The most active compound for females was dihydrocarvone, and the response was compared to that obtained for total OAV, followed by limonene, butyl hexanoate, 4-ethyl acetophenone, and caryophyllene oxide. Antennae of females gave a significantly much higher signal than did males ($P < 0.05$) to dihydrocarvone, 4-ethyl acetophenone, carvone, and total OAV. The most active compounds for males were limonene, limonene oxide, dihydrocarvone, butyl hexanoate, and total OAV.

TABLE 2. ANALYSIS OF VARIANCE[a]

VS	SS	*dF*	MS	*F* ratio
P_i	116,542.64	12	9,711.89	58.67[b]
C‖	179,321.69	5	35,864.34	695.18[b]
S_k	8.18	1	8.18	0.05
$F_{l(ij)}$	17,215.64	104	165.54	3.21[b]
$(P \times C)_{ij}$	40,186.64	60	669.78	12.98[b]
$(P \times S)_{ik}$	16,832.17	12	1,402.68	8.47[b]
$(C \times C)_{jk}$	1,872.58	5	374.52	7.26[b]
$(P \times C \times S)_{ijk}$	9,667.20	60	161.12	3.12[b]
$(C \times F)_{jl(ik)}$	26,826.56	520	51.59	
Total	408,473.30	779		

[a]VS: variation source, SS: sum of squares, *dF*: degrees of freedom, MS: mean square, P: product, C: concentration, S: sex, F: fly.
[b]Significance level = 0.01.

DISCUSSION

Limonene is the most abundant compound in both orange essential oil and in the OAV and is a constituent of the volatiles released by males of *C. capitata* (Jang et al., 1989). Another major compound in OAV is alloaromadendrene, which has not been described in the orange essential oil but is present in that of *Sideritis hirsuta* (Mateo et al., 1983) and others (Connolly and Hill, 1991). Butyl and ethyl hexanoate found in OAV at intermediate levels have been described from orange essential oil.

Some other chemicals found in OAV at very low concentrations (limonene oxide, menta-2,8-dien-1-ol, dihydrocarvone, carveol, carvone, β-copaene, γ-bisabolene, cadinene, caryophyllene oxide, and nootkatone) are minor components in the orange essential oil (McLeod et al., 1988; Sugiswa et al., 1989; Schreier et al., 1977), 1-Isopropyl-4-methylbenzene is a component of the essential oil of *Catasetum* and *Clowesia* (Knudsen et al., 1993), ethyl acetophenone occurs in *Ophrus insectifera* L. y *O. speculum* Link (Knudsen et al., 1993), aromadendrene is found in *Eucaliptus delegatensis* (Connolly and Hill, 1991), cascarilladiene is in present *Guisa aromatica* (Claude-Lafontaine, et al., 1976), glogulol occurs in *Eucaliptus globulus*, sphatulenol is present in *Eucaliptus sphatulata* (Connolly and Hill, 1991), and 1(10)-aristolen-2-one is found in *Aristolochia longa* (De Pascual et al., 1983). Clovene and 2,6-di-t-butyl-1,4-benzoquinone have not been described as components of plant aromas.

1,2-Diethylbenzene and 1,3-diethylbenzene, which occur at intermediate concentrations, may be artifacts of Porapak Q, although they have been described

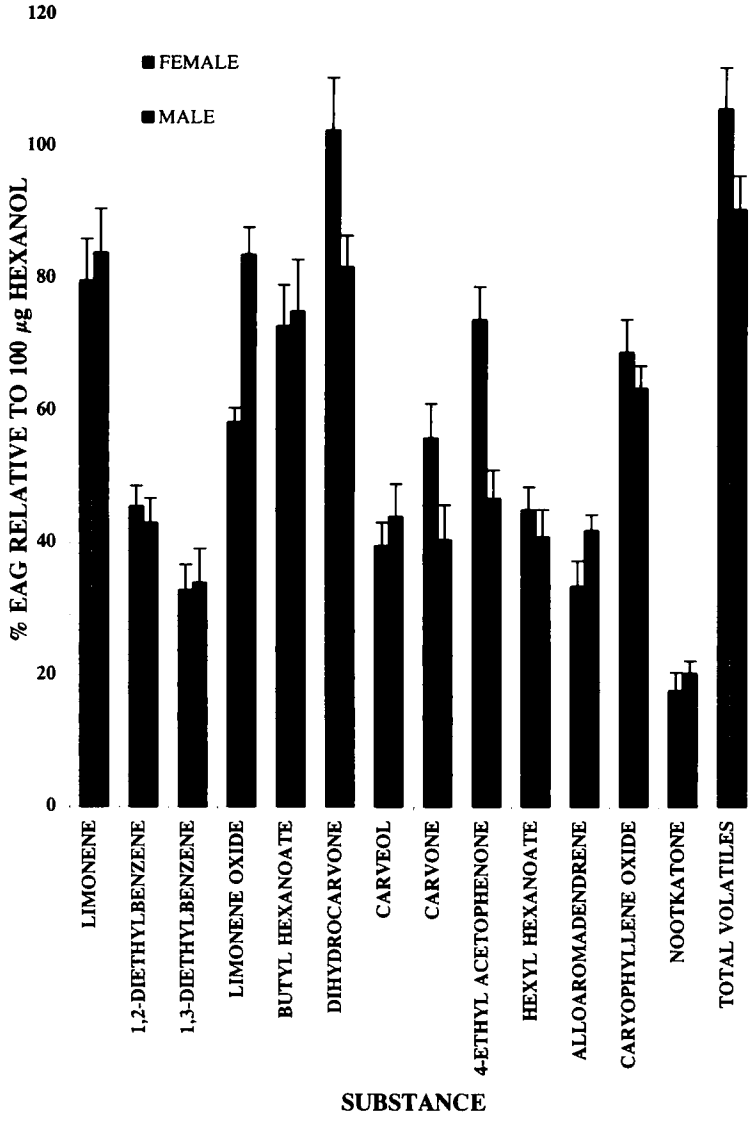

FIG. 3. Mean EAG optimal responses from antennae of *C. capitata* females and males to the compounds identified in fresh orange airborne volatiles. The dose represented is 1000 μg for all compounds, except 4-ethyl acetophenone and carveol in females (100 μg). Vertical lines show the ± standard error ($N = 5$). A 100% response approximately corresponds to 8 mV.

as constituents of the essential oil of *Syringa* (Knudsen et al., 1993). Dihydrocarvone, 4-ethyl acetophenone and carvone produced a much higher response from females than from males. The most active compounds to females were limonene, 4-ethyl acetophenone, butyl hexanoate, dihydrocarvone, and caryophyllene oxide. Jang et al. (1989) found that ethyl acetate, geranyl acetate, (*E*,*E*)-α-farnesene, myrcene, linalyl acetate, ethyl butyrate, nonyl acetate and (*E*)-β-farnesene, all isolated from volatiles released by males, elicited larger EAGs in females than in males. Light et al. (1988) found that females have a higher sensitivity than males for 1-undecanol, (*E*)-2-buten-1-ol, (±)-2-heptanol, (±)-1-octen-3-ol, hexanal, nonyl acetate, decyl acetate, and decanoic acid. The compounds ethyl acetate, methyl octanoate, myrcene, (*Z*)-3-hexen-1-ol, β-felandrene, ethyl butyrate, hexanal, and δ-decalactone identified in the essential oil of nectarines elicited higher responses in females (Light et al., 1992).

The most active compounds in the EAG for both males and females were limonene (one of the most abundant in orange volatiles), butyl hexanoate (intermediate concentration in the volatiles), and dihydrocarvone, limonene oxide, and caryophyllene oxide (low concentrations in the volatiles). There does not seem to be a relationship between the concentration of a compound in the volatiles and the magnitude of the EAG response. This agrees well with the results obtained by Light et al. (1992) in nectarines.

The total OAV elicited larger EAGs, females being more sensitive than males. There were no significant differences between the response to dihydrocarvone and total OAV. Levinson et al. (1990) assayed the essential oil of some fruits by EAG and obtained the highest responses to orange, lemon, and bergamot. Higher signals were obtained in females than in males in all instances.

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QUANTITATIVE COMPARISON OF VOLATILE COMPOUNDS AMONG SEVEN *Medicago* Spp. ACCESSIONS

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Abstract—Previous research identified the presence of unique semivolatile compounds in alfalfa weevil (*Hypera postica* Gyllenhal) (AW) -resistant *Medicago* accessions. These compounds may impart repellency to AW in alfalfa (*Medicago sativa* L.). The objective of our study was to analyze within- and between-population variation for four of these unique semivolatile compounds across eglandular and glandular-haired *Medicago* accessions. Seven *Medicago* accessions were evaluated under greenhouse conditions in an experiment arranged as randomized complete blocks with four replicates. Volatile compounds were extracted via supercritical fluid extraction and then quantitatively analyzed for levels of 2,7-dimethyl, 2,6-octadienol; *cis*-geraniol; 1,(*E*)-8,(*Z*)-10-tetradecatriene; and 1-hepten-3-ol. Significant difference ($P < 0.05$) among entries were observed for 2,7-dimethyl,2,6-octadienol; *cis*-geraniol; 1, and (*E*)-8,(*Z*)-10-tetradecatriene. These compounds were found only in glandular-haired accessions. There were no significant differences in levels of 1-hepten-3-ol. Our findings demonstrate that the genetic incorporation of 2,7-dimethyl, 2,6-octadienol; *cis*-geraniol; 1, and (*E*)-8,(*Z*)-10-tetradecatriene into cultivated alfalfa will require interspecific crossing. Alfalfa weevil behavioral tests using these compounds must be conducted prior to such crosses in order to ascertain the efficacy of repellency by these compounds.

Key Words—*Medicago* spp., alfalfa, volatiles, grandular hairs, insect repellents, *Hypera postica* Gyllenhal, germplasm.

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INTRODUCTION

The Egyptian and western strains of alfalfa weevil (AW) are serious economic pests of alfalfa in the western United States of America (Hsiao, 1993). Because no AW-resistant cultivars exist (Hodgson and Posler, 1986) and economic losses are high (Kennedy et al., 1987), other means have been employed to curb this injurious pest. Early harvest, chemical control, and biological control have all been attempted with only marginal success. (Hsiao, 1993; Danielson et al., 1987b; Keith et al., 1982; Manglitz and Ratcliffe, 1988; Moffett et al., 1986; Anonymous, 1984).

Researchers have identified a number of wild *Medicago* species exhibiting significant AW resistance (Barnes and Ratcliffe, 1969; Johnson et al., 1980a,b; Shade et al., 1975). In some cases, nonpreference was attributed to deterrence caused by erect glandular hairs present on leaves and stems of wild *Medicago* spp. Trichome density has also been suggested, but not correlated, as an agent of repellency (Danielson et al., 1986, 1987a,b; Johnson et al., 1981). Other studies have focused on plant volatile compounds, in addition to trichomes, as contributors to antixenosis (Core et al., 1994; Doss et al., 1989, Doss and Johnson, 1991).

Core et al. (1994) isolated and tentatively identified 97 plant volatile and semivolatile compounds from three wild *Medicago* species and five alfalfa cultivars. Of these 97 compounds, 17 were qualitatively unique to AW-resistant, glandular-haired *Medicago* species. Three of the 17 compounds were observed in relatively high concentrations. If these volatiles compounds are found in high concentrations in glandular-haired *Medicago* species exhibiting alfalfa weevil repellency, they may represent biologically active compounds. Furthermore, if significant variation for these compounds is present in alfalfa cultivars, then it may be possible to increase selectively the emanation of those volatiles exhibiting biological activity. Otherwise, interspecific hybridization and selection will be required to introgress volatiles from the wild species into cultivated types. The objective of our study is to analyze within- and among-population variation in tissue content for four unique semivolatile compounds among eglandular and glandular-haired *Medicago* accessions.

METHODS AND MATERIALS

Four volatile compounds [1-hepten-3-ol (HEPT); *cis*-geraniol (GERA); 2,7-dimethyl,2,6-octadien-1-ol (OCTA); 1,(*E*)-8, (*Z*)-10 tetradecatriene (TETR)], tentatively identified by Core et al. (1994), were analyzed for quantitative variation among seven *Medicago* accessions consisting of five cultivars, Caliverde, Caliverde 65, CUF101, Moapa 69, and CADL, and two germplasm

sources, KS94GH6 (*M. glandulosa* David) (Sorensen et al., 1986) and KS108GH5 (*M. glutinosa* Marshall von Bieberstein) (Sorensen et al., 1985). Accessions were planted in a glasshouse as randomized complete blocks with four replicates. Leaf and stem tissue from five individual plants per accession were sampled from each replicate and subjected to supercritical fluid extraction as described by Henning et al. (1994). Supercritical fluid extraction was utilized due to its proven efficiency in extraction and recovery of volatiles often lost by other procedures such as headspace sampling and Soxhlet extraction (Hawthorne et al., 1988; Henning et. al., 1994). Using this procedure allowed for a more accurate assay of all volatile compounds produced by the plant, both precursor and emitted volatiles.

A Hewlett Packard Series II model 5890 gas chromatograph with flame ionization detection capabilities fitted with an Alltech Associates, Inc. Econocap, 30-m × 0.24-mm-ID capillary column (0.25-μm coating of SE-54) was used for compound separation. Temperature and flow parameters were as follows: carrier (helium) velocity = 21 cm/sec, makeup gas (nitrogen) flow = 30 ml/min, initial oven temperature = 45°C for 5 min, temperature program ramp = 5°C/min, final temperature = 200°C for 5 min. We injected 2 μl of sample using a splitless injection for 1.2 min followed by split purge with a split of 100:1. The inlet temperature was maintained at 215°C, while the detector was set to 250°C.

Quantitative analyses of the four volatile compounds were determined based on their relationship to the peak height of an internal standard (36.5 μg of dodecane), which was added to every sample. An external standard of dodecane was assayed every 10 samples. Peak areas for the four compounds were transformed to represent equivalent micrograms of dodecane. All data are thus reported as microgram equivalents of dodecane per gram of plant material.

The data were analyzed as a split plot where accessions were considered as whole plots and genotypes within accessions as subplots. Replicates and accessions were considered as random effects. Analysis of variance using least-squares procedures (Snedecor and Cochran, 1980) were computed on the data using Statistix v.4.0 analytical software.

RESULTS AND DISCUSSION

We selected HEPT for analysis in this study because in a previous study it was qualitatively the most abundant compound, appearing in all GC chromatograms across all *Medicago* germplasm sources (Core et al., 1994). The other three compounds were chosen because: (1) they were qualitatively observed in a limited sample consisting of three plants representing three different wild *Medicago* species, and (2) their peaks were readily discernible on a GC chro-

TABLE 1. MEAN SQUARES FROM ANALYSIS OF VARIANCE FOR HEPT, OCTA, GERA, AND TETR IN CALIVERDE, CALIVERDE 65, CUF101, MOAPA 69, CADL, KS94GH6 (*M. glandulosa* DAVID), AND KS108GH5 (*M. glutinosa* MARSHALL VON BIEBERSTEIN)

		Compound (ng equivalents of dodecane/g plant material)			
Source	*df*	HEPT	OCTA	GERA	TETR
Blocks (B)	3	8551.54[a]	0.39037	0.11066	3.88033
Germplasm (G)	6	2578.50	2.86552[b]	3.86806[b]	7.41456[a]
B * G	18	2390.32[b]	0.27217	0.14687	2.27241
Error	108	642.610	0.37401	0.22682	1.41567

[a]Significant at $P \leq 0.05$.
[b]Significant at $P \leq 0.01$.

matogram. The relative abundance of these threse semivolatile compounds, however, appeared to be far less than the relative abundance for HEPT (Core et al., 1994).

We observed significant differences ($P < 0.05$) between seven germplasms for GERA, OCTA, and TETR (Table 1). Mean separation analysis using Fisher's least significant difference (LSD) (Snedecor and Cochran, 1980) identified KS94GH6 as possessing significantly higher levels ($P < 0.01$) of GERA and OCTA than the other six germplasm sources (Table 2). KS94GH6 possessed

TABLE 2. AVERAGE INTERNAL CONCENTRATION FOR LEVELS OF HEPT, OCTA, AND TETR IN SEVEN ALFALFA GERMPLASM ACCESSIONS [CALIVERDE, CALIVERDE 65, CUF101, MOAPA 69, CADL, KS94GH6 (*M. glandulosa* DAVID), AND KS108GH5 (*M. glutinosa* MARSHALL VON BIEBERSTEIN)].

	Nanogram equivalents of dodecane/g plant material[a]			
Germplasm	HEPT	OCTA	GERA	TETR
Caliverde	30.455a	0.0733a	0.0081a	0.0000a
Caliverde 65	31.680a	0.2213a	0.0000a	0.0000a
CUF101	56.548a	0.1074a	0.0212a	0.0098a
Moapa 69	48.658a	0.1409a	0.0358a	0.0000a
CADL	26.355a	0.2610a	0.0104a	0.0176a
KS108GH5	32.687a	0.1573a	0.0273a	0.6593ab
KS94GH6	29.355a	1.1472b	1.1802b	1.5871b
LSD	32.482	0.3466	0.2546	1.0015

[a]Means for a specific compound with similar letters are not significantly different from one another based upon LSD ($P < 0.05$).

significantly higher levels of TETR than did the alfalfa cultivars with the exception of KS108GH5. Our data indicate that GERA, OCTA, and TETR are not present or are found in extremely low levels in the *M. sativa* cultivars we tested. There were no significant differences among entries for HEPT (Table 1). Levels of HEPT were at 100- to 200-fold higher levels than any of the other three compounds.

Both qualitative and quantitative results indicate that volatile components occur in AW-resistant KS94GH6 and KS108GH5 germplasm, which are either not present, or are present in extremely low levels, in the susceptible alfalfa cultivars examined. These data suggest that attempts to develop alfalfa cultivars possessing high levels of these compounds will require interspecific crosses. Following interspecific crossing, several cycles of selection for increased expression of these compounds must be practiced while selecting against undesirable traits coincorporated with these speculative repellent compounds.

If these compounds were expressed in nontrichome tissue, then the next question is whether or not they are genetically correlated. We did observe a strong phenotypic correlation ($P \leq 0.01$) between GERA and OCTA (Table 3). This phenotypic correlation between GERA and OCTA suggests (but does not prove) a relationship between these two compounds based potentially on biosynthetic pathways. *cis*-geraniol is one of the monoterpenes produced directly from geranyl pyrophosphate (GPP) (Goodwin and Mercer, 1983) and as such may contribute to the production of OCTA, or OCTA may be formed directly from GPP or neryl pyrophosphate, another monoterpene precursor. The compounds TETR and HEPT were not correlated with each other or with either of the two terpenes (GERA and OCTA). This is what would be expected due to the dissimilarities in their respective biosynthesis pathways.

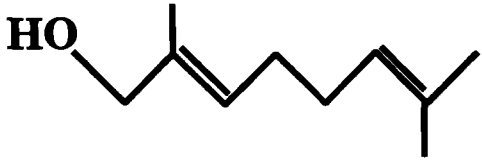
GERA ($C_{10}H_{18}O$) is an isomer of isogeraniol, differing only in the placement of one double bond (Figure 1). Isogeraniol was isolated from AW extracts

TABLE 3. PHENOLYTIC CORRELATIONS BETWEEN HEPT, OCTA, GERA, AND TETR IN CALIVERDE, CALIVERDE 65, CUF101, MOAPA 69, CADL, KS94GH6, (*M. glandulosa* DAVID), AND KS108GH5 (*M. glutinosa* MARSHALL VON BIEBERSTEIN)

Compound	OCTA	TETR	GERA	HEPT
OCTA	1.00			
TETR	0.3414	1.00		
GERA	0.8675[a]	0.3725	1.00	
HEPT	0.0933	−0.2138	−0.0153	1.00

[a] Significant at $P < 0.01$.

cis-geraniol $C_{10}H_{18}O$



2,7-dimethyl-2,6-octadienol $C_{10}H_{18}O$

FIG. 1. Chemical structures of *cis*-geraniol and 2,7-dimethyl-2,6-octadienol (Core et al., 1994).

and may be a possible pheromone for this insect although its exact function is unknown (Hedin et al., 1988). The other two volatile compounds, OCTA and TETR, may function as secondary plant compounds involved in AW repellency due to their singular presence in glandular-haired repellent species. Behavioral assays will be necessary to determine the attractancy or repellancy of these compounds to alfalfa weevils.

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VARIATION IN APHID ALARM PHEROMONE CONTENT AMONG GLANDULAR AND EGLANDULAR-HAIRED *Medicago* ACCESSIONS[1]

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Abstract—Pea (*Acyrthosiphon pisum* Harris) and blue alfalfa aphid (*A. kondoi* Shinji) deterrency in alfalfa (*Medicago sativa* L.) may result from incorporating higher levels of the aphid alarm pheromone, (*E*)-β-farnesene relative to (*E*)-β-caryophyllene. We evaluated five eglandular and two glandular-haired alfalfa accessions for differences in (*E*)-β-farnesene and (*E*)-β-caryophyllene content under glasshouse conditions using supercritical fluid extraction and gas chromatography. In addition, pea and blue alfalfa aphid olfactory behavioral tests were conducted upon *Medicago* species containing different ratios of (*E*)-β-farnesene relative to (*E*)-β-caryophyllene. No differences in (*E*)-β-caryophyllene content were observed among the seven entries (μ = 0.42 ng/g plant material). Significant differences ($P \leq 0.05$) among entries were observed for (*E*)-β-farnesene content, with KS94GH6 exhibiting the highest (1.18 ng/g), and CUF 101 the lowest levels (0.35 ng/g). Elite tetraploid sources possessed significantly lower levels (μ = 0.42 ng/g) of (*E*)-β-farnesene than did wild and cultivated diploid accessions (μ = 0.83 ng/g). Olfactory behavioral tests for both the pea and blue alfalfa aphids demonstrated KS94GH6 repelled aphids while cultivated alfalfa types attracted aphids in each case. Previously demonstrated aphid resistance in diploid KS94GH6 may result from superior (*E*)-β-farnesene levels, but (*E*)-β-farnesene is probably not a factor in cultivated alfalfa resistance. Finally, accession KS94GH6 could act as an excellent germplasm resource for the incorporation of higher (*E*)-β-farnesene levels into cultivated alfalfa.

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Key Words—Alfalfa, *Medicago sativa Acrythosiphon pisum*, *Acyrthosiphon kondoi*, (*E*)-β-farnesene, (*E*)-β-caryophyllene, glandular-hairs, aphid resistance.

INTRODUCTION

Pea aphids and blue alfalfa aphids cause significant economic losses in alfalfa. A combination of pesticides and resistant cultivars is currently used to control infestations. Pesticides registered for use on alfalfa have been curtailed due to tighter government regulations and high registration or reregistration costs. Resistance in most alfalfa cultivars is due to antibiosis (Manglitz and Ratcliffe, 1988). In some cases, antibiosis resistance has broken down as strong selection for adapted aphid biotypes has taken place (Nielson et al., 1971). Additional resistance mechanisms should be incorporated into elite germplasms in conjunction with antibiosis to impede the development of resistant aphid biotypes.

Plant resistance due to nonpreference, when coupled with antibiosis, may reduce selection pressure and ultimately prolong plant resistance. Ferguson et al. (1982) described one mechanism in which aphid nonpreference was thought to be due to chemical signals in glandular-haired *Medicago* spp, but the signals responsible for nonpreference were not identified, Gibson and Pickett (1983) identified (*E*)-β-farnesene as the chemical cue for aphid (*Myzus persicae* Sulzer) nonpreference in *Solanum berthaultii* Hawkes. (*E*)-β-Farnesene, a sesquiterpene, is the alarm pheromone for *M. persicae* and is also the alarm pheromone for pea aphids and a number of other economically important aphid species (Nault et al., 1973; Pickett and Griffiths, 1980).

Research by Dawson et al. (1984) suggested that aphid repellency due to the aphid alarm pheromone could be overcome by the presence of sufficient (*E*)-β-caryophyllene, another sesquiterpene found in glandular hairs of *Solanum tuberosum* L.. This group observed that as ratios of (*E*)-β-farnesene to (*E*)-β-caryophyllene were reduced below 3:1, aphids were less likely to be repelled. Ratios greater than 3:1 of (*E*)-β-farnesene to (*E*)-β-caryophyllene resulted in aphid repellency.

Buttery et al. (1982) identified both (*E*)-β-farnesene and (*E*)-β-caryophyllene as minor volatile components of alfalfa foliage. We hypothesize that it might be possible to reduce pea and blue alfalfa aphid feeding preference by increasing the quantities of (*E*)-β-farnesene relative to (*E*)-β-caryophyllene. This alteration may be achieved through interspecific crosses with germplasms known to exhibit aphid repellency. Prior to attempting such crosses, it would be desirable to know: (1) if sufficient levels of (*E*)-β-farnesene relative to (*E*)-β-caryophyllene were present in cultivated alfalfa, or if higher levels might be obtained through introgression from aphid-repellent wild perennial species, and (2) if nascent levels of (*E*)-β-farnesene relative to (*E*)-β-caryophyllene in cultivated

alfalfa and aphid-repellent germplasm are sufficient to repel both blue alfalfa aphids and pea aphids.

METHODS AND MATERIALS

Quantification of Plant Volatiles. Seven plant entries consisting of five alfalfa varities, CUF 101, Caliverde, Caliverde 65, Moapa 69, CADL, and two *Medicago* germplasms, KS94GH6 (Sorensen et al., 1986) and KS108GH5 (Sorensen et al., 1985), were planted in soil beds (mixed, thermic Typic Torripsamments) in a glasshouse at Las Cruces, New Mexico. Glasshouse conditions were: 30°C ± 5°C (day), 25°C ± 5°C (night); and 16L:8D photoperiod. The seven entries were selected based on their levels of resistance or susceptibility to blue alfalfa and pea aphids (Table 1). CADL was chosen based upon its ability to produce fertile offspring from crosses with diploid KS94GH6. The experiment was arranged as a randomized complete block design with four replicates. Sources of variation for the experiment were: individual plants (five plants per entry per replicate), entry (seven entries), and replicates (four replicates). Entry plots were spaced 1 m apart. Plant individuals were considered nested within entries. Entries were considered fixed, while replicates and individual plants within entries were considered as random factors.

Leaf and stem tissue samples were collected and processed according to the procedures reported by Core et al. (1994). Plant volatile compounds were extracted via supercritical fluid extraction (SFE) as described by Henning et al. (1994) using a Hewlett-Packard model 7680A (Avondale, Pennsylvania).

TABLE 1. LEVEL OF RESISTANCE TO BLUE ALFALFA APHIDS (BAA; *Acyrthosiphon kondoi* SHINJI) AND PEA APHIDS (PA; *Acyrthosiphon pisum* HARRIS)[a]

Germplasm	Resistance level[b]	
	BAA	*PA*
CUF101	HR	HR
Moapa 69	NA	S
Caliverde	S	S
Caliverde 65	S	S
CADL	NA	NA
KS108GH5	R	R
KS94GH6	HR	HR

[a] As reported in the Alfalfa Catalog (Townsend et al., 1994).
[b] HR = high resistance (>50% of population is resistant), R = resistant (31–50% of population is resistant), S = susceptible (0–5% of population is resistant, NA = information not reported).

Peak identification and cochromatographic verification of (*E*)-β-farnesene and (*E*)-β-caryophyllene from plant tissues were determined on a gas chromatograph–mass spectrophotometer (GC-MS) (Saturn model, Varian Inc.; Sugarland, Texas) with an ion-trap detector. The GC-MS conditions were the same as those published by Henning et al. (1994). Quantification of (*E*)-β-farnesene and (*E*)-β-caryophyllene content was accomplished with a Hewlett-Packard model 5890 Series II gas chromatograph (GC) with external standards of (*E*)-β-farnesene (20.78 ng/μl), (*E*)-β-caryophyllene (20.78 ng/μl), and docanal (internal standard) at 18.25 ng/μl.

Gas chromatography was carried out with the initial GC oven temperature set at 45°C for 5 min. The oven temperature was then increased 5°C/min to a final temperature of 200°C, resulting in a total run time of 41 min. The GC operating conditions were: injector temperature, 215°C; detector temperature, 250°C; 2 μl splitless injection, split turned on after 1.2 min; helium carrier flow rate, 1 ml/min; nitrogen makeup flow rate, 30 ml/min; hydrogen flow rate, 45 ml/min; and compressed air flow rate, 350 ml/min.

The data were analyzed initially by analysis of variance. Bartlett's test for variance heterogeneity (Snedecor and Cochran, 1980) revealed significant differences among entry variances. As a result, post hoc mean comparisons among germplasms were accomplished through the use of Student's *t* test (Snedecor and Cochran, 1980).

Aphid Behavioral Studies. Pea and blue alfalfa were obtained from R. Barberet and A. Zarrabi (Department of Entomology, Oklahoma State University) and were maintained in two separate growth chambers to ensure purity of lines for testing. Blue alfalfa aphids were maintained under a 16L:8D photoperiod, 20°C, and 50% relative humidity, while pea aphids were maintained under similar conditions, but at 23°C temperature. Aphids were denied food (*Medicago sativa* L. cv CUF 101) for 2 hr prior to testing. This length of time was determined to be optimum for aphid behavioral activity.

The linear track olfactometer used in our study was based upon the design reported by Brewer et al. (1983) and consisted of a transparent Plexiglass Y tube fitted with recepticles designed to hold either 2 g of plant material or moistened filter paper. A purified, humidified airflow of 2.5 liters/min/arm was utilized in all behavioral tests. Aphids were prevented from viewing recepticles containing the test materials by means of black construction paper. Thus, the only means of attraction for aphids consisted of volatile compounds either present (plant material) or absent (moistened filter paper).

Plant materials used in behavioral assays were maintained in the greenhouse under optimum growth conditions. Five entries were bioassayed. Four of the entries were selected from the experimental line COMP88, representing four ratios of (*E*)-β-farnesene relative to (*E*)-β-caryophyllene: i.e., high–high, high–low, low–high, and low–low (Table 2). We used the aphid-resistant germplasm

TABLE 2. AVERAGE CONCENTRATION OF (*E*)-β-FARNESENE AND (*E*)-β-CARYOPHYLLENE AND RATIO BETWEEN COMPOUNDS IN FOUR PREVIOUSLY SELECTED RATIO TYPES OF ALFALFA (*Medicago sativa* L.) AND KS94GH6 (*M. sativa* var. *viscosa*)

Plant type	Concentration (ng/g[a])		Ratio
	(*E*)-β-Farnesene	(*E*)-β-Caryophyllene	
Low-high[b]	0.345	0.533	0.65:1
Low-low	0.260	0.245	1.06:1
High-high	0.490	0.400	1.23:1
High-low	0.505	0.285	1.77:1
KS94GH6	1.180	0.430	2.75:1

[a] Nanograms compound per gram of plant material.
[b] Ratio of (*E*)-β-farnesene to (*E*)-β-caryophyllene.

KS94HG6 (Sorensen et al., 1986) as the fifth entry due to its high level of (*E*)-β-farnesene relative to (*E*)-β-caryophyllene (Table 2). Levels of (*E*)-β-farnesene and (*E*)-β-caryophyllene were determined as reported above.

Tests consisted of introducing 10 aphids into the Y tube, sealing the end, and allowing aphids 30 min for choice of arm. At the end of this time, the number of aphids choosing either the air arm or the plant arm were recorded and then removed. The Y tube was then washed in 95% ethanol, dried, and utilized for the next set of experiments. For blue alphafa aphids, a total of 80 aphids was used for each of the plant entries with the exception that 20 aphids were used for KS94GH6. The same number of aphids were used for pea aphid tests. Student's *t* tests (Snedecor and Cochran, 1980) were utilized to test for differences between entries within aphid species. No tests were run for differences between aphid species.

RESULTS AND DISCUSSION

We observed significant differences among germplasms for (*E*)-β-farnesene concentration levels ($P = 0.005$). The germplasm source, KS94GH6 (*M. glandulosa*) exhibited the highest concentration (1.17 ng/g plant material) of (*E*)-β-farnesene among all entries (Table 3). None of the tetraploid alfalfa cultivars (CUF101, Moapa 69, Caliverde, and Caliverde 65) differed in their content of (*E*)-β-farnesene. There appeared to be a trend ($P \leq 0.05$) with the wild-type or diploid germplasm accessions (KS94GH6, CADL, and KS108GH5) containing significantly higher levels of (*E*)-β-farnesene ($\mu = 0.83$ ng/g) than the tetraploid cultivars ($\mu = 0.42$ ng/g). We also observed significant differences

TABLE 3. AVERAGE CONCENTRATION, RANGE OF VALUES, AND POPULATION STANDARD DEVIATION FOR (*E*)-β-FARNESENE AMONG SEVEN *Medicago* ENTRIES INCLUDING KS108GH5 (*M. glutinosa*) AND KS94GH6 (*M. sativa* var. *viscosa*)

Germplasm	Concentration (ng/g)[a]		
	Germplasm Average	Range	Standard Deviation
CUF101	0.35 a[b]	0.00–0.94	0.32 a[c]
Moapa 69	0.40 a	0.00–1.25	0.39 ab
Caliverde	0.40 a	0.00–1.16	0.41 ab
Caliverde 65	0.51 a	0.00–2.14	0.52 bc
KS108GH5	0.53 a	0.00–2.91	0.71 cd
CADL	0.79 ab	0.00–2.58	0.80 d
KS94GH6	1.18 b	0.00–6.14	1.80 e

[a] Nanograms (*E*)-β-farnesene per gram plant material.
[b] Means followed by the same letter are not significantly different based on *t* test ($P \leq 0.05$).
[c] Standard deviations followed by the same letter are not significantly different ($P \leq 0.05$) based on Bartlett's test for heterogeneity of variances (Snedecor and Cochran, 1980).

among germplasm sources for within-population variances (Table 3), with KS94GH6 exhibiting the greatest variation followed by CADL and KS108GH5. Not surprisingly, the cultivated alfalfa entries exhibited the lowest within-population variances.

There were no differences between entries for concentration of (*E*)-β-caryophyllene (Table 4). Mean concentration of (*E*)-β-caryophyllene ranged from

TABLE 4. AVERAGE CONCENTRATION OF (*E*)-β-CARYOPHYLLENE AMONG SEVEN *Medicago* ENTRIES INCLUDING KS108GH5 (*M. aglutinosa*) AND KS94GH6 (*M. sativa* var. *viscosa*)

Germplasm	Germplasm average (ng/g)[a]
CUF 101	0.40 a[b]
Moapa 69	0.41 a
Caliverde	0.35 a
Caliverde 65	0.50 a
KS108GH5	0.42 a
CADL	0.46 a
KS94GH6	0.43 a

[a] Nanograms (*E*)-β-farnesene per gram plant material.
[b] Means followed by the same letter are not significantly different based on *t* test ($P \leq 0.05$).

0.35 to 0.50 ng/g plant material. In addition, there were no differences between population variances for this compound (data not shown). These results suggest that selection for decreased concentration of (*E*)-β-caryophyllene may not be possible due to a potential lack of genetic variation in the alfalfa populations we studied.

We observed significant differences in blue alfalfa aphid attraction to the five entries studied for aphid behavior (Figure 1). There was a significant trend towards repellency with increasing ratios of (*E*)-β-farnesene to (*E*)-β-caryophyllene ($y = 97.318 - 21.024 * x$, $P = 0.0102$). KS94GH6, with a ratio of 2.75 : 1 [(*E*)-β-farnesene to (*E*)-β-caryophyllene], which appears to repel aphids as more aphids chose the air arm over the plant arm. Thus, it appears that near-sufficient levels of (*E*)-β-farnesene relative to (*E*)-β-caryophyllene are present in KS94GH6, while insufficient levels of (*E*)-β-farnesene relative to (*E*)-β-caryophyllene were found in cultivated alfalfa types. Thus, KS94GH6 holds promise as a means for introducing higher levels of (*E*)-β-farnesene relative to (*E*)-β-caryophyllene into cultivated alfalfa.

Similar results were observed for behavioral tests using pea aphids, with

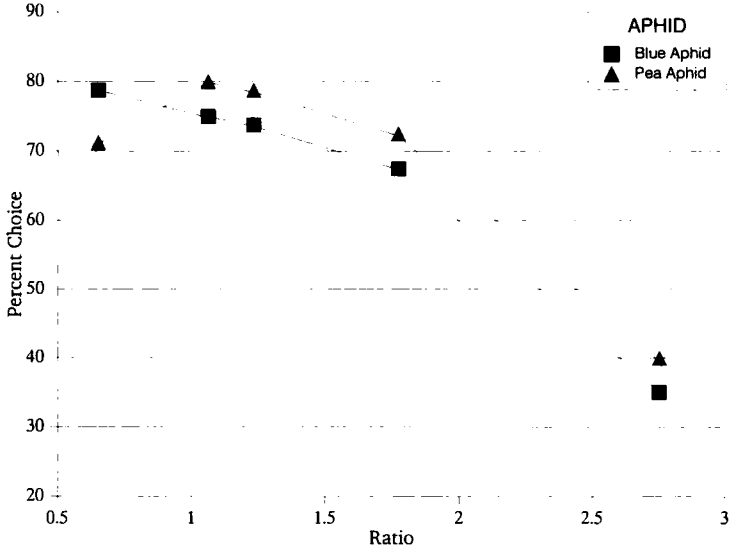

FIG. 1. Relative percentage of blue alfalfa aphid (*Acyrthosiphon kondoi* Shinji) and pea aphid (*Acyrthosiphon pisum* Harris) choosing olfactometer arm with *Medicago* species plant volatiles (*y* axis = aphid choice) as a function of ratio of (*E*)-β-farnesene to (*E*)-β-caryophyllene (*x* axis = ratio).

KS94GH6 repelling pea aphids while all alfalfa entries attracted aphids (Figure 1). The analysis of variance for regression of aphid choice on ratio was not significant ($P = 0.0732$). The most probable cause for this result may be that the aphids from one replication of the alfalfa entry for low–high (0.65 : 1 ratio) inadvertently were tested too soon after feeding, resulting in no choice by all 10 aphids. This reduced the number of potential plant arm choices for the 0.65 : 1 ratio to levels below that of the other three alfalfa types. Nonetheless, all four alfalfa types attracted aphids, while the KS94GH6 repelled aphids.

Sorensen et al. (1986) described KS94GH6 as possessing high resistance to pea and blue alfalfa aphids. Our data indicate that part of this resistance may be due to high concentrations of (*E*)-β-farnesene relative to (*E*)-β-caryophyllene (2.75 : 1). The cultivar CUF101, however, also exhibited high resistance to both pea and blue alfalfa aphids (Townsend et al., 1994), but it possessed significantly lower levels of (*E*)-β-farnesene and exhibited a lower ratio of (*E*)-β-farnesene relative to (*E*)-β-caryophyllene (0.88 : 1) than did KS94GH6. Resistance in CUF101 appears to be due to another mechanism, possibly antibiosis.

One potential method to increase the levels of (*E*)-β-farnesene emitted by plant tissue is to increase the internal concentration of this compound relative to (*E*)-β-caryophyllene. The fact that genetic variation for (*E*)-β-caryophyllene may not be present, coupled with the apparent lack of correlation between (*E*)-β-caryophyllene and (*E*)-β-farnesene concentration, may allow single-trait selection for increased (*E*)-β-farnesene levels, particularly since (*E*)-β-farnesene was present in all *M. sativa* entries. Our results suggest that higher internal (*E*)-β-farnesene levels may be incorporated through genetic integression with KS94GH6 as a donor parent. Some of the problems encountered by such a procedure are requirements for (1) scaling up from a diploid to a tetraploid during the initial cross, and (2) numerous back-crosses to cultivated alfalfa to eliminate undesirable characteristics contributed from KS94GH6.

The ultimate goal in increasing internal (*E*)-β-farnesene levels is to increase the release of this alarm pheromone compound while maintaining low levels of (*E*)-β-caryophyllene. Montgomery and Nault (1978) demonstrated that headspace concentrations (the concentration of compound immediately surrounding the plant) of (*E*)-β-farnesene in excess of 0.1 ng were necessary to repel *Myzus persicae*. The internal plant concentration of (*E*)-β-farnesene in KS94GH6 ranged from 0.00 to 6.14 ng/g (Table 2). Further studies were necessary to determine if these higher values are sufficient to generate threshold headspace values greater than 0.1 ng.

Simply increasing the internal (*E*)-β-farnesene content of alfalfa may not produce the desired repellency, but Dawson et al. (1984) determined that increasing the relative concentration of (*E*)-β-farnesene to (*E*)-β-caryophyllene from 1 : 3 to 10 : 3 significantly increased aphid dispersal of *Lipaphis erysimi* L. and *Phorodon humulis* S. Our behavioral work suggests similar threshold

requirements for both the pea and blue alfalfa aphids. Nevertheless, additional work is required to determine the actual threshold ratios for these two compounds to repel both pea and blue alfalfa aphids. Once this ratio is known, accurate goals can be set regarding the internal levels of (*E*)-β-farnesene relative to (*E*)-β-caryophyllene necessary to condition aphid dispersal.

CONCLUSION

There are no published reports on the presence of genetic variation for (*E*)-β-farnesene in *Medicago* species. Interspecific crosses with accessions such as KS94GH6 are one means of increasing the internal concentration of this compound in alfalfa. Once higher levels of (*E*)-β-farnesene are introduced into an autotetraploid background, repeated cycles of selection for individuals exhibiting superior levels of (*E*)-β-farnesene relative to (*E*)-β-caryophyllene may condition aphid repellency in alfalfa. The genetic incorporation of higher levels of (*E*)-β-farnesene into alfalfa cultivars already possessing antibiosis may help to impede the breakdown of plant resistance to aphids.

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GRAIN WEEVIL, *Sitophilus granarius* (L.): ANTENNAL AND BEHAVIORAL RESPONSES TO MALE-PRODUCED VOLATILES

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Abstract—Coupled GC-EAG techniques have been applied to the study of volatiles from the grain weevil, *Sitophilus granarius*, for the first time. The size of EAG response was independent of the sex of the responding insect but was consistently larger to extracts of males than those of females. This difference was reflected in a behavioral preference for the male extracts by mated adults of both sexes tested together and virgin adults of both sexes tested separately. The GC-EAG results provide evidence for two materials that are released specifically by the males. Using circular dichroism, one has been found to be identical stereochemically with the (2*S*,3*R*)-sitophilate reported by others as the aggregation pheromone in a different strain. This enhances the prospects for the development of a single pheromone lure that would be generally applicable whatever the origin of the strain. The small amount of sitophilate found in the males suggests that it is not stored in large amounts. The other material, present in such a small amount that it has yet to be fully characterized, elicits a higher antennal activity than sitophilate and may have a significant role to play in enhancing the trap catch of this economically important pest.

Key Words—*Sitophilus granarius*, grain weevil, Coleoptera, aggregation pheromone, electroantennogram, coupled GC-EAG, behavioral bioassay, circular dichroism.

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INTRODUCTION

The grain, or granary, weevil, *Sitophilus granarius* (L.), is a primary storage pest, being able to attack previously undamaged grains. It can infest wheat, barley, maize, various other cereals, and cereal products. Although it is ecologically suited to more temperature climates, it is found all over the world and is spread by trade. Presently, it is one of the most important storage pests in the UK (Prickett, 1988; Prickett and Muggleton, 1991) and causes serious nutrient and economic damage. The development of its immature stages, unseen within grain kernels, means that its presence may go unnoticed until serious damage has been caused and emphasizes the need for improved detection: typically, traps in grain may catch less than 1% of the *S. granarius* present (Cogan and Wakefield, 1994).

Faustini et al. (1982) presented evidence for a male-produced *S. granarius* aggregation pheromone that was attractive to both sexes. This has since been identified as 1-ethylpropyl (2*S*,3*R*)-3-hydroxy-2-methylpentanoate [trivial name (2*S*,3*R*)-sitophilate] (Phillips et al., 1987, 1989). Laboratory tests of its effectiveness in the presence of wheat have been conducted (Plarre, 1994) but there have so far been no examples of its practical application in a trap lure.

To enhance trap catch in practical situations, it is essential to gain a detailed understanding of semiochemicals such as pheromones. Previous work has demonstrated the contribution to such an understanding from the combined study of antennal and behavioral responses of other storage beetles (White and Chambers, 1989; White et al., 1989; Chambers et al., 1990) and the differences in behavioral response to pheromone components shown by different strains of the same species (Chambers et al., 1990).

The aim of the present work was to apply a combined study of antennal and behavioral responses of *S. granarius* to male-produced volatiles to help understand their role in coordinating behavior.

METHODS AND MATERIALS

General. S. granarius (Windsor strain), cultured at an average population density of 500 insects per 50 g wheat grain (CV Avalon) and at 25°C and 70% relative humidity, was used for all experiments. All glassware was washed and then heated at 470°C for 8 hr before use. Solvents were of HPLC grade and, in addition, dichloromethane was eluted through Al_2O_3 (neutral, Brockmann grade 1) and stored in darkness at 10°C.

Collection of Insect Volatiles. Volatiles from individual insects of known age and sex were collected by a method adapted from Phillips et al. (1985). Individual grains from an insect culture were checked for infestation by nuclear

magnetic resonance (Chambers et al., 1984). Infested grains were placed individually in glass tubes (5 × 2.5 cm diameter) coated on the inside near the top with Fluon (an aqueous suspension of polytetrafluoroethylene from Whitford Plastics Ltd., Runcorn, UK). The tubes were stored at 25°C and 70% relative humidity and capped. They were vented for about 5 min three times each week to provide sufficient oxygen.

The sex of newly emerged adults was determined on the basis of their abdominal and rostral characteristics (Halstead, 1963). Each insect was placed in a clean tube with one wheat grain and a filter paper disk (Whatman grade 1, 2 cm diameter). The disks and grains were changed weekly over eight weeks. Each week's disks were pooled for each sex (200 males and 200 females) and extracted with 200 ml dichloromethane for at least 24 hr, giving extracts 1–8M and 1–8F. (Abbreviations for each extract and its origin are given in Table 1.) Similarly, 200 tubes each containing a filter paper disk and an uninfested wheat grain cut transverse to the crease, were maintained under standard conditions for two weeks. Two weekly extracts were made, as above, but were pooled to give extract W.

For a second batch, the volatiles of 210 males (extracts 1′–8′M) and 109 females (extracts 1′–8′F) were collected for eight weeks. In addition, frass was collected from the tubes after eight weeks, and the males were cut at the thorax–elytra junction, bisected to give two body sections for extraction. These were the heads together with prothoraxes, and rest of thoraxes with the abdomens. The separate frass from the males and females and the male body sections were

TABLE 1. IDENTITY OF EXTRACTS

Label	Origin
1–8M, 1–8F	Dichloromethane extract of 200 disks from tubes with males and wheat grains (M) or females and wheat grains (F) for one of eight consecutive weeks (1–8) after emergence
W	Dichloromethane extract of volatiles from 200 uninfested cut wheat grains for two weeks
1′–8′M, 1′–8′F	Repeat of first extraction experiment using 210 males (M) and 109 females (F)
Fr_m, Fr_f	Dichloromethane extract of frass of males (m) or females (f) collected after the repeat extraction experiment
H + P, T + A	Dichloromethane extract of heads and prothoraxes (H + P) or thoraxes and abdomens (T + A) of males used in the repeat extraction experiment
$Mf_{1\text{–}4}$, $Ff_{1\text{–}4}$	Preparative gas chromatography fractions (1–4) of pooled weekly extracts of males (M) or females (F) from the first extract experiment

extracted with 200 ml dichloromethane each, giving extracts Fr_m, Fr_f, H + P, and T + A, respectively. All extracts were reduced to 1 ml by rotary evaporation at 30–35°C (bath) and 16 mm Hg, then stored at −20°C.

Electroantennography. The electroantennogram (EAG) system was similar to that described previously (White et al., 1989). The recording and indifferent electrodes were inserted into the terminal antennal segment and the ventral surface of the head, respectively. Test cartridges consisted of 40 × 4-mm filter paper strips impregnated with the test material and placed inside Pasteur pipets, from which an airstream at 1000 ml/min in 1-sec pulses took volatiles to a delivery tube. The outlet of the delivery tube was aligned along the antennal axis at a distance of approximately 10 mm, which ensured that the whole antenna was within the airstream. To ensure that odors were removed after presentation, an exhaust system was suspended over the preparation. Recordings were made using intact individuals. A total of five male and five female beetles two to six weeks after emergence were tested in each experiment.

To determine whether adult beetles produced any EAG-active volatiles, the EAG responses to the first batch of extracts were studied. Each beetle was presented with 10 μl of 1M–6M and 1F–6F in randomized order. In a further experiment beetles were presented with 10 μl of 2M, 2F, and W volatiles. Results were analyzed by split-plot ANOVA.

In a third EAG experiment, to determine which of (2*S*,3*R*)- and (2*R*,3*S*)-sitophilate produced the greater EAG response, synthetic samples (Mori and Ishikura, 1989) were assayed at 10^{-1} ng to 10^4 ng to give dose–response curves. The EAG activities of the two diastereomers of the closely related racemic 1-ethylpropyl 3-hydroxy-2-methylbutanoate (separated by preparative GC) were recorded at doses from 10^{-1} to 10^3 ng.

Pitfall Bioassays. The level of attraction to extracts was measured using a two-choice pitfall test (White and Birch, 1987). Test insects were conditioned for 24 hr without food and in darkness. Ten insects were introduced into each arena and allowed 1 hr to settle. Test and control stimuli (5 μl) were then applied to 2-cm-diameter filter paper disks supported on wire grids to aid solvent evaporation. Grids were used once only to prevent cross-contamination. The dichloromethane solvent was allowed to evaporate for at least 1 min, the disks were placed in pitfall tubes, and the arenas covered with glass plates. After 1 hr the numbers of insects in the test and control tubes were counted. All tests were set up and run under red light at 25°C and 70% relative humidity during mornings. The insects were tested only once and then discarded.

Two sets of tests were undertaken. In the first, mated adults of both sexes combined, three to five weeks since emergence, were given choices between 2M and dichloromethane, 2F and dichloromethane, W and dichloromethane, or 2M (test) and W (control). In the second set of tests, virgin adults of the separate sexes, 10 weeks ± 1 day after emergence, were given choices between 2M and

dichloromethane or 2F and dichloromethane. Six replicates of each test were conducted using a randomized design.

The results were subjected to binomial logistic analysis, first for the proportion of insects responding (insects found in either pitfall), then for the directional nature of the response by comparing the numbers of insects in the test and control pitfall tubes (Chambers et al., 1990).

Coupled Gas Chromatography–Electroantennogram (GC-EAG) Procedure. Extracts were analyzed using a coupled GC-EAG technique (White and Birch, 1987). Extracts 2M, 2F, and W were analyzed using a 50-m × 0.32-mm-ID OV-1 column (Phase Separations Ltd.) with helium as a carrier gas and an oven temperature held at 50°C for 1 min, then programmed to 220°C at 10°C/min. Injections were made in the splitless mode with the injector at 220°C and the flame ionization detector (FID) at 240°C. The antennal responses of a female were recorded twice to each of 2M and 2F; the responses of three males were recorded in all 12 times to 2M, and five times to each of 2F and W. The same system was later fitted with a 50-m × 0.25-mm-ID CPSil5CB column (Chrompack) to compare a combined male extract (Mf_2, see later for preparative GC method) with the two diastereomers of racemic 1-ethylpropyl 3-hydroxy-2-methylbutanoate and to compare extracts 2′M and 6′M with synthetic (2*S*,3*R*)-sitophilate. Oven temperature programs were varied. The antennal responses of a female were recorded three times for Mf_2 and once for each of a mixture of Mf_2 and each of the diastereomers. The responses of a second female were recorded once for 2′M, three times for 6′M, and once for (2*S*,3*R*)-sitophilate.

Capillary Gas Chromatography (GC). Comparison of extract 2M with (4*R*,5*R*)- and (4*S*,5*R*)-sitophilure [synthesized by Mori and Ebata (1986)] was conducted using a 50-m × 0.25-mm-ID CPSil5CB column (Chrompack) with splitless injection; injector 220°C; FID 280°C; oven 50°C for 1 min then to 220°C at 10°C/min. Extracts Fr_m, Fr_f, H + P, T + A, 1′M–8′M, Mf_3, and synthetic (2*S*,3*R*)-sitophilate were analyzed on a 50-m × 0.25-mm-ID CPSil19CB column (Chrompack) with splitless injection; injector 220°C; FID 280°C; oven 50°C for 1 min then to 220°C at 5°C/min.

Coupled Gas Chromatography–Mass Spectrometry (GC-MS). Extract 2M was analyzed with a Hewlett-Packard 5790 GC coupled to a Jeol JMS-DX300 mass spectrometer. Samples were introduced by splitless injection (injector 230°C) onto a 25-m × 0.22-mm-ID BP-1 column with helium as carrier gas. Oven programs were varied, and column effluent was ionized by electron impact (EI) at 70 eV and chemical ionization (CI) with ammonia. Fractionated pooled weekly extracts Mf_2 and Ff_2 (see later for preparative GC method) were analyzed on the same system using a 50-m × 0.25-mm-ID CPSil5CB column (Chrompack); injector 220°C; oven 50°C for 1 min then to 220°C at 5°C/min. This system, fitted with a 50-m × 0.25-mm-ID CPSil19CB (Chrompack) column, with the injector temperature at 230°C and an oven program from 40°C for 1

min then to 270°C at 10°C/min, was also used to compare a synthetic mixture of all four stereoisomers of 1-ethylpropyl 3-hydroxy-2-methylbutanoate with Mf_2. For these extracts the eluent was ionized solely by electron impact at 70 eV.

Preparative Gas Chromatography. The first batch of extracts was pooled for each sex and fractions collected after elution through a 2-m × 2-mm-ID column of 3% OV-101 on Chromosorb W-HP 100–120 mesh; injector 220°C; thermal conductivity detector 240°C; filament temperature 290°C; filament current 211 mA. Helium was used as carrier gas at 30 ml/min, and the oven was programmed from 50°C to 130°C at 3°C/min then to 220°C at 20°C/min. Four fractions were collected for both male and female extracts (Mf_{1-4} and Ff_{1-4}, respectively) to separate different components which showed EAG activity.

Crude 1-ethylpropyl 3-hydroxy-2-methylbutanoate was purified using the same system with an oven program of 50°C to 120°C at 1°C/min. Two fractions (diastereomers 1 and 2) representing the two enantiomeric pairs were collected.

Circular Dichroism (CD) Spectroscopy. Solutions of synthetic (2*S*,3*R*)- and (2*R*,3*S*)-sitophilate and Mf_3 (containing sitophilate) were analyzed with a Jasco J600 Spectropolarimeter. The synthetic solutions were made up to 2.5×10^{-2} mol/dm^3 in acetonitrile and scans taken from 285 to 185 nm at a 0.05-cm pathlength with a time constant of 4 sec and bandwidth of 2 nm. The extract solution was more dilute at 1.2×10^{-4} mol/dm^3 in acetonitrile and 10 scans taken at a 1-cm pathlength were averaged.

Synthesis of 1-Ethylpropyl 3-hydroxy-2-methylbutanoate. 3-Pentanol (40 ml, 0.37 mol) and pyridine (16.2 ml, 0.2 mol) were mixed in a 250-ml round-bottom flask and 2-bromopropionyl chloride (17 ml, 0.17 mol) was added slowly. The reaction mixture was stirred at 75°C for 4 hr and then filtered into 50 ml water. After shaking, the water was discarded and the organic layer washed successively with three 50-ml aliquots of 1 mol/dm^3 aqueous copper sulfate, 50 ml water, 50 ml 1 mol/dm^3 sodium hydrogen carbonate, and 50 ml brine. After drying over calcium sulfate, the product was distilled and 1-ethylpropyl 2-bromopropionate (0.1 mol, 59% yield) collected at 1 mm Hg between 73.5 and 75.5°C. (60 MHz ^{1}H NMR, $CDCl_3$: ppm relative to TMS, splitting pattern, relative intensity, assignment: 0.9, triplet, 0.40, 2 $-CH_3$; 1.6, quintet, 0.26, 2 $-CH_2-$; 1.7, doublet, 0.21, $-CH_3$; 4.4, quartet, 0.06, CH—Br; 4.8, quintet, 0.06, CH—O—.)

This bromo ester was converted to the required β-hydroxybutyric ester by a Reformatski reaction procedure (Rathke and Lindert, 1970). Zinc powder (unactivated, 3.27 g, 0.05 mol) was put into a 100-ml round-bottom flask which was then flushed with nitrogen and sealed with a rubber septum. Tetrahydrofuran (13 ml), acetaldehyde (2.8 ml, 0.05 mol) and trimethylborate (13 ml) were injected through the septum, and stirring of the mixture commenced. The

1-ethylpropyl 2-bromopropionate (9 ml, 0.04 mol) was injected and the mixture maintained at 40°C. After 4 hr a mixture of glycerol (14 ml) and ammonium hydroxide (pH 13, 14 ml) was added, and after shaking the organic phase was separated. The aqueous layer was extracted with three 15-ml aliquots of diethyl ether, which were added to the organic phase. The combined organic phase was washed with 100 ml brine. Vacuum distillation between 54 and 74°C and 1 mm Hg yielded 0.02 mol unreacted bromo ester and a residue that was not distilled. This residue was shown to be 1-ethylpropyl 3-hydroxy-2-methylbutanoate [MS, EI: 36(27%), 43(45%), 55(65%), 70(25%), 74(100%), 83(15%), 85(7%), 101(85%), 144(5%), 159(4%)] in a yield of 25% (0.01 mol). (60 MHz ^{1}H NMR, $CDCl_3$: ppm relative to TMS, splitting pattern, relative intensity, assignment: 0.9, triplet, 0.29, 2 $-CH_3$; 1.0–1.4, overlapping multiplets, 0.31, $-CH_3$ and $-CH_3$; 1.6, quintet, 0.23, 2 $-CH_2-$; 2.5, multiplet, 0.07, $-CH-CO_2-$; 4.4, multiplet, 0.05, $-C\underline{H}-OH$; 4.8, quintet, 0.05, $-CH-O-$.)

RESULTS

In the EAG analysis of extracts 1–6M and 1–6F, there was no difference between the response of the two sexes ($F = 1$, NS), so the data have been combined (Table 2). There was no consistent increase or decrease in response to the six weekly male or female extracts. Extracts from males produced significantly larger EAG responses than female extracts, in both males and females ($P < 0.001$). In the comparison of extracts 2M, 2F (week 2 having been chosen arbitrarily), and W (Table 3), the response to 2M was significantly greater ($P < 0.001$) than to either 2F or W, and the response to W was significantly greater ($P < 0.05$) than to 2F (t test).

In the first set of pitfall bioassay tests there was no significant difference between the different treatments in activity (the probability of responding to either of the two pitfalls taken together; $\chi^2 = 7.22$, $P > 0.05$). The fitted probability of responding was 0.81 (95% confidence intervals 0.70, 0.88). However, of those insects that did respond, there was a significant difference between the numbers responding to the test pitfall as opposed to the control pitfall ($\chi^2 = 13.86$, $P < 0.005$). The fitted probabilities of responding to the test pitfall are presented in Table 4. Significantly more insects entered the pitfall containing 2M, 2F, and W when these were paired with solvent and the pitfall containing 2M when paired with W. In the second set of pitfall bioassay tests, there was no significant difference between the number of virgin males and females responding to either of the two pitfalls but there were significant differences between the treatments ($\chi^2 = 8.3$, $P < 0.005$). In general, more insects responded in the 2M vs. dichloromethane test than in the 2F vs. dichloromethane

TABLE 2. EAG RESPONSES OF 10 INDIVIDUALS (5 MALES AND 5 FEMALES) TO 10 μl OF EXTRACTS 1–6M AND 1–6F

Extract number	Mean response (μV ± SE)	
	Male extract (M)	Female extract (F)
1	1430 ± 160	670 ± 100
2	1640 ± 260	740 ± 80
3	1300 ± 160	540 ± 80
4	1280 ± 160	610 ± 80
5	1530 ± 210	640 ± 60
6	1350 ± 180	590 ± 90
Source	*df*	*F*
Sex of responder (SR)	1	1
Residual 1	8	
Sex of producer (SP)	1	222.7[a]
Age of producer (AP)	5	2.5[b]
SR × SP	1	1.8
SR × AP	5	0.9
SP × AP	5	0.5
SR × SP × AP	5	1.3
Residual	88	
Total	119	

[a] $P < 0.001$.
[b] $P < 0.05$.

TABLE 3. EAG RRESPONSES OF 10 INDIVIDUALS (5 MALES AND 5 FEMALES) TO 10 μl OF EXTRACTS 2M, 2F, AND W

Extract number	Mean response (μV ± SE)	
2M	1440 ± 180	
2F	660 ± 80	
W	890 ± 160	
Source	*df*	*F*
Sex of responder (SR)	1	0.2
Residual 1	8	
Extract tested	2	42.2[a]
Sex × extract tested	2	2.0
Residual	16	
Total	29	

[a] $P < 0.001$.

TABLE 4. PITFALL BIOASSAY RESULTS WITH MATED ADULTS OF BOTH SEXES TESTED TOGETHER; PROBABILITY OF RESPONDING TO TEST PITFALL AS OPPOSED TO CONTROL PITFALL

Treatment: test vs. control	Fitted probability[a]	95% Confidence intervals	
		Upper	Lower
2M vs dichloromethane	0.88	0.95	0.74
2F vs dichloromethane	0.72	0.84	0.57
W vs dichloromethane	0.72	0.84	0.56
2M vs W	0.94	0.98	0.83

[a]Equal response to the two pitfalls would be represented by a probability of 0.50.

test (Table 5A). The virgin males and females differed in the directional nature of the response (Table 5B). In general, more females responded to the test pitfalls than males, and there was a greater response to 2M than to 2F.

Analysis of extract 2M by GC-EAG showed four areas of EAG activity (Figure 1): component A, retention time (R_t) between 11.89 and 12.11 min; component B R_t 12.91 min; component C R_t 13.15 min; component D R_t 13.91 min. Component A gave the largest EAG response but no identifiable FID signal. The female extract contained only components B and D. Extract W showed no areas of EAG activity by GC-EAG.

GC-MS of extract 2M suggested that component C was aliphatic [MS, EI: 43(39%), 55(34), 59(27), 69(18), 74(100), 85(12), 97(8), 103(24), 115(45),

TABLE 5. PITFALL BIOASSAY RESULTS WITH VIRGIN ADULTS OF BOTH SEXES TESTED SEPARATELY

Treatment: test vs. control	Sex of responder	Fitted probability	95% Confidence intervals	
			Upper	Lower
A. Probability of responding to either test or control pitfall				
2M vs. dichloromethane		0.94	0.98	0.84
2F vs. dichloromethane		0.83	0.91	0.70
B. Probability of responding to test pitfall as opposed to control pitfall				
2M vs. dichloromethane	Male	0.70	0.85	0.48
2M vs. dichloromethane	Female	0.93	0.99	0.72
2F vs. dichloromethane	Male	0.51	0.72	0.32
2F vs. dichloromethane	Female	0.65	0.84	0.43

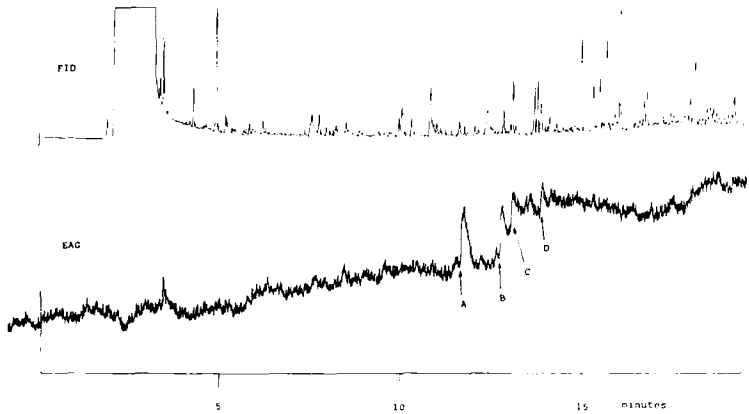

FIG. 1. GC-EAG response from a female *S. granarius* to extract 2M from males showing the position of components A–D.

144(6), 173(3); MS, CI: molecular ion of 202] and MS data were consistent with those of sitophilate (Phillips et al., 1987). Component C was collected in the fractionated pooled weekly extract Mf_3. CD spectral data of natural sitophilate in Mf_3 (λ_{max} = 212 nm, Δe = positive), synthetic (2*S*,3*R*)-sitophilate (λ_{max} = 212 nm, Δe = +0.3), and (2*R*,3*S*)-sitophilate (λ_{max} = 212 nm, Δe = −0.3) suggested that the natural material is the 2*S*,3*R* stereoisomer. In the EAG dose–response tests, there were no significant differences between the responses of the two sexes, so the data have been combined. Figure 2a shows that the 2*S*,3*R* stereoisomer is significantly more active than the 2*R*,3*S* stereoisomer. In the capillary GC comparison, the amount of sitophilate detected increased from none in 1′M to less than 1 μg in each of 2′M and 3′M, and to more than 2.5 μg in each of 4′M to 8′M. No sitophilate was found in Fr_f, but Fr_m contained 7 μg (equivalent to less than 1 μg collected per week). T + A contained 0.6 μg sitophilate whereas none was detected in H + P.

Identification of component A was also attempted because of its high EAG activity and because it was present in only the male extracts. Comparison by capillary GC showed that synthetic (4*R*,5*R*)- and (4*S*,5*R*)-sitophilure (R_t 8.5 and 8.6 min, respectively) eluted before component A in extract 2M (R_t approximately 10 min). GC-MS of extract 2M gave no structural information about component A as there was insufficient material. Preparative GC of the pooled weekly extracts provided more material for analysis. In the region where component A would be expected to elute, GC-MS analysis of male extract Mf_2 showed an unknown [MS, EI, 36(63%), 43(53%), 55(95%), 70(33%), 74(100%), 83(10%), 85(14%), 101(55%), 144(2%), 159(2%); MS, CI: molec-

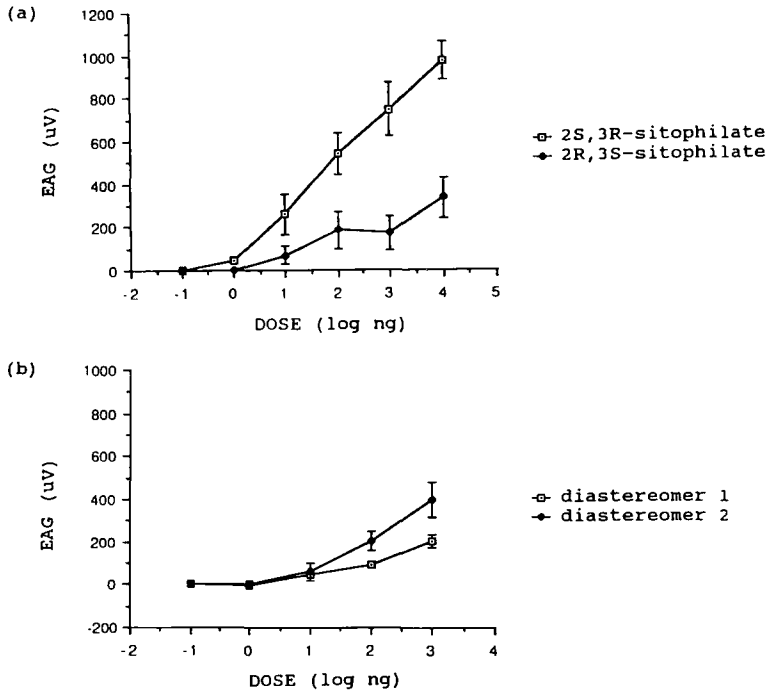

FIG. 2. EAG dose–response curves of ten *S. granarius* adults, five of each sex, to (2*S*,3*R*)- and (2*R*,3*S*)-sitophilate (a), and to diastereomers 1 and 2 of 1-ethylpropyl 3-hydroxy-2-methylbutanoate (b).

ular ion of 188]. This unknown was not found in female extract Ff_2. Comparison with the MS data for sitophilate suggested that the unknown might be 1-ethylpropyl 3-hydroxy-2-methylbutanoate. This compound was synthesized and GC-MS showed that it coeluted with the unknown and the MS data were identical for both.

EAG dose–response curves for diastereomers 1 and 2 of racemic 1-ethylpropyl 3-hydroxy-2-methylbutanoate (Figure 2b) showed low activity for diastereomer 2 and significantly lower activity for diastereomer 1 when compared with EAG response to (2*S*,3*R*)-sitophilate (Figure 2a). GC-EAG analysis of Mf_2 and mixtures of Mf_2 with either diastereomer showed (Table 6A) that diastereomer 2 has a different retention time from component A and that the coelution of diastereomer 1 with component A does not cause a proportional increase in the EAG response to component A. The ratios of the EAG and FID responses in Table 6A show that component A has higher relative EAG activity than either

TABLE 6. RELATIVE EAG AND GC (FID) RESPONSES OF MATERIALS ELUTING IN THE REGION OF VARIOUS COMPONENTS

Injection	R_t (min)	FID signal[a]	EAG signal[a]	EAG/FID ratio	Comments
A. Component A in male extract Mf_2 and diastereomers 1 and 2 of 1-ethylpropyl 2-methyl-3-hydroxybutanoate					
Mf_2	24.4	30	35	1.2	Component A
Mf_2	23.9	30	25	0.8	Component A
Mf_2 + Diast.1	24.2	75	20	0.3	Diast.1 + component A
Mf_2 + Diast.2	24.4	23	25	1.1	Component A
	24.7	93	24	0.3	Diastereomer 2
B. Components A and C in male extracts 2'M and 6'M and synthetic (2*S*,3*R*)-sitophilate					
2'M	24.2	3	6	2.0	Component A
	26.9	25	25	1.0	Component C
6'M	24.2	5	12	2.4	Component A
	26.9	70	26	0.4	Component C
(2*S*,3*R*)-Sitophilate	26.9	40	30	0.8	Sitophilate

[a]Arbitrary units.

of the diastereomers. Thus 1-ethylpropyl 3-hydroxy-2-methylbutanoate is not the highly EAG-active component A. Comparison of 2'M, 6'M, and (2*S*,3*R*)-sitophilate by GC-EAG (Table 6B) confirms that the relative activity of component A is higher than component C and that the activity at component C is associated with sitophilate.

DISCUSSION

There was no difference between the two sexes in the size of EAG response to the volatiles tested, whether they were of synthetic or natural origin. In comparison with other storage pests, the saw-toothed grain beetle, *Oryzaephilus surinamensis*, also shows no sexual differences (White and Chambers, 1989), although the females of two *Cryptolestes* species show larger EAG responses than the males (Chambers et al., 1990). These results may reflect sexual dimorphism in antennal structure of the *Cryptolestes* species. Details of the antennal structure of *Sitophilus granarius* have not yet been established, although some interesting branched sensilla have been observed (D. B. Pinniger, personal communication). Our results with the sitophilate isomers are comparable with those of Levinson et al. (1990) from a different strain of *S. granarius*.

The consistently greater EAG response to the male extracts than the female extracts implies that the release of male-produced EAG-active volatiles remains

essentially constant over the first six weeks after their emergence. The EAG response to female extract 2F was probably smaller than to wheat extract W because by the end of each week, the females had consumed most of the wheat grain. The extracts made in the seventh and eighth weeks were not tested by EAG although they were pooled with the other extracts for fractionation by preparative gas chromatography.

The results of the two-choice pitfall tests were separated into activity (whether the insects respond to either pitfall) and directional nature of the response (whether responders are found in the test or control pitfall) (Chambers et al., 1990) to overcome the inadequacy of the commonly used formula: (test − control)/number tested. This separation showed that with the mated insects the differences in response between treatments arose solely from the different directional effects of the extracts rather than any differences in their ability to stimulate a response. The results demonstrate the behavioral importance of the male-produced volatiles. Although male-produced volatiles from the saw-toothed grain beetle showed no sexual differences in EAG response, marked differences were found in behavioral effects, with a greater response from females than males (White and Chambers, 1989). To investigate whether this might also be the case with *S. granarius*, a second set of behavioral tests was conducted using virgin males and females tested separately. Differences were found, with the females giving a greater directional response than the males, but the differences were not statistically significant.

This is the first time that coupled GC-EAG studies have been reported for *S. granarius* and their usefulness is clearly demonstrated by rapidly locating two volatiles with EAG activity produced only by the males, components A and C, the former not having been reported previously. Further, the fact that the wheat extract showed no specific areas of activity by GC-EAG shows that its EAG activity when presented as a mixture must be due to small contributions from a large number of components. This would appear to be typical of the response of storage pests to food aromas.

Component C has the same gross structure as the sitophilate found in a different strain of *S. granarius* (Phillips et al., 1987). The novel application of circular dichroism confirmed that the strain studied here produces the 2*S*,3*R* isomer of sitophilate, like the strain studied by Phillips et al. (1989). This is reflected in the behavioral effects of the 2*S*,3*R* and 2*R*,3*S* isomers, comparable threshold doses for attractancy being 10 and 100 ng, respectively (McGreevy et al., 1993). Analysis of the frass extracts confirmed that sitophilate production is confined to the males, and the small amount in these extracts relative to those in the extracts of the filter paper disks suggested that its presence in the frass was merely due to some slight absorption on it rather than being its source. The small amount found in the thorax and abdomen extract of the males suggests that sitophilate is not stored in large amounts.

It was thought at first that component A, the other material with EAG activity produced specifically by the males, might be sitophilure, which has been found in trace amounts in extracts of *S. granarius* (Schmuff et al., 1984). (4*S*, 5*R*)-Sitophilure is the trivial name given to (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone, the aggregation pheromone of *S. zeamais* and the rice weevil, *Sitophilus oryzae* (Schmuff et al., 1984; Walgenbach et al., 1987). It has been reported that both sexes of *S. granarius* are attracted to volatiles collected from male *S. zeamais* (Walgenbach et al., 1983) and synthetic racemic sitophilure (Phillips et al., 1985), which would imply that there should be some EAG response. However, in the present study no evidence has been found for either diastereomer of sitophilure in the extracts of volatiles from *S. granarius*.

The EAG activity of component A is readily observable, yet its FID response is very small and correct assignment is complicated by the presence of other materials with similar retention times. The discovery of a male-specific material in this region with similar mass spectral characteristics to sitophilate suggested that the identity of component A should be the analogous butanoate. Comparison with synthesized material, however, demonstrated that neither diastereomer has the right EAG characteristics. The butanoate is not an obvious degradation product of the pentanoate, sitophilate, so is unlikely to have been formed during isolation and probably is produced by the males. It may be formed as a secondary metabolite by a similar pathway to sitophilate but from different starting materials. It is nevertheless curious that its GC elution characteristics should be so similar to the still unidentified component A.

It is particularly interesting that component A should have a noticeably higher EAG response per unit FID response than sitophilate. It would be surprising if it did not have some important significance for *S. granarius*. In preliminary tests to compare an extract of males containing 10 ng (2*S*,3*R*)-sitophilate with 10 ng of this sitophilate isomer alone, females responded significantly more ($P < 0.05$) to the former. This confirms the behavioral activity of materials in the extract other than sitophilate. This activity may be sexually specific since males showed no significant difference in this comparison. The generally greater behavioral response of virgin females compared with virgin males to isolates of natural volatiles from *S. granarius*, observed both here and in earlier work (Faustini et al., 1982), contrasts with the observation of almost identical response of the two sexes to synthetic (2*S*,3*R*)-sitophilate (Phillips et al., 1989) and might be explained by the presence of component A in the natural isolates. Laboratory tests with synthetic (2*S*,3*R*)-sitophilate in the presence of wheat have shown that its function is more that of an arrestant than an attractant (Plarre, 1994), which suggests that the function of component A might be complementary to that of the sitophilate in guiding insects to its source. If this is so, then component A would have a valuable role to play in enhancing trap catch.

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HEMICELLULOSE IS AN IMPORTANT LEAF-FEEDING RESISTANCE FACTOR IN CORN TO THE FALL ARMYWORM[1]

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Abstract—The fall armyworm, *Spodoptera frugiperda* (J. E. Smith), (FAW) is a major pest of corn, *Zea mays* L., in the southeastern United States. The damage to pretassel corn is caused by larvae feeding primarily on immature inner whorls. In this study, resistant lines were found to contain more crude fiber in whorls, mostly hemicellulose and cellulose. While hemicellulose, chiefly an arabinoxylan, was higher in resistant (R) lines than in susceptible (S) lines, the distribution of constituent neutral sugars was very similar in the lines. Both lines also contained *p*-coumaric and ferulic acids. These phenolic acids are known to occur both in the free state and in the cell wall as complexes bound by ester linkages to the arabinose moiety of the arabinoxylan. ^{13}C NMR data showed that the intensity of the carbonyl carbon (184 ppm) in resistant hemicellulose was stronger, indicating a greater degree of cross-linking. Thus, resistant hemicellulose is both structurally different from susceptible hemicellulose and present in greater quantities. In two of three laboratory dietary tests, FAW larval weight gains were significantly higher on diets with (S) hemicellulose incorporated at the same level as (R) hemicellulose. Therefore, resistance to the FAW appears to be correlated with both a greater amount and a higher degree of cross-linking of the hemicellulose of (R) lines.

Key Words—Corn, *Zea mays* (L.), fall armyworm, *Spodoptera frugiperda* (J. E. Smith), Lepidoptera, feeding resistance, hemicellulose.

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INTRODUCTION

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith), (FAW) is a major pest of corn, *Zea mays* L., in the southeastern United States (Davis et al., 1988a,b). The damage to pretassel corn is caused by larvae feeding primarily on inner whorl leaf tissue. The damage caused by this insect can be extensive; in fact, late-planted corn is sometimes destroyed.

A long-continuing corn breeding program at Mississippi State, Mississippi, has led to the release of eight germplasm lines with leaf-feeding resistance to the FAW and the southwestern corn borer (SWCB), *Diatraea grandiosella* Dyar (Davis et al., 1988a,b). These lines have also shown resistance to the sugarcane borer, *Diatraea saccharalis* (Fab.), the corn earworm, *Heliothis zea* (Boddie), and the European corn borer (ECB), *Ostrinia nubilalis* (Hubner) (Davis et al., 1988b). The resistance appears to be operating by the same mechanisms (antibiosis and nonpreference), suggesting that the same or similar chemical and physical factors may govern the resistance of corn to these insects (Wiseman et al., 1981, 1983; Williams et al., 1985, 1987; Davis et al., 1988a,b).

Two major qualitative factors of resistance in corn to one or several corn insects are 6-MBOA–DIMBOA (Klun and Brindley, 1966) and maysin (Waiss et al., 1979). However, our investigations demonstrated that either these compounds possessed only limited toxicity to the FAW or SWCB or that they were present at minor levels in the corn lines found to have significant resistance to these two insects (Hedin et al., 1984, 1990, 1993). Resistance to a leaf disease in corn was also attributed to lignin by Lyons et al. (1993), but we could only find 1.5% lignin in susceptible corn whorls and 1.6% lignin in resistant whorls (Hedin et al., 1984).

Waxes may also contribute to FAW resistance. The surfaces waxes of FAW-resistant lines were reported to be thicker (Davis et al., 1995). Earlier, Hedin et al., (1993) found the yield of waxes averaged 0.047% and 0.069% from fresh tissue for (S) and (R) lines, respectively. GLC and GLC-MS analyses revealed only minor differences in the composition of the waxes, but 6-MBOA, DIMBOA, and *N-O*-Me-DIMBOA were present in higher concentrations in (R) lines. However, these constituents are known not to be very toxic to the FAW and SWCB (Hedin et al., 1993). Thus, any contribution to resistance by the waxes is probably related to physical attributes that may modify the behavior of the larvae (Yang et al., 1991, 1992).

Because qualitative defenses such as DIMBOA do not appear to contribute significantly to resistance of whorls to the FAW and the SWCB, quantitative defenses by cell wall constituents should be considered. The cell wall of plants has been characterized as consisting of cellulose microfibrils embedded in a lignin–hemicellulosic macromolecule to which acetyl and phenolic acid groups

are bound (Morrison, 1979). Arabinoxylans are the characteristic hemicellulose of cereals (Fry, 1989).

The phenolic constituents of the cell walls of graminaceous plants consist largely of *p*-coumaric and ferulic acids (Hartley and Jones, 1978; Sosulski et al., 1982). These acids are bound to cell wall polysaccharides through ester bonds (Fry, 1986). Based on polysaccharide hydrolase digests of sugar cane and corn cell walls, ferulic acid was shown to be linked to arabinose, which in turn is linked to xylose (Kato and Nevins, 1985). Classen et al. (1990) concluded that cell wall-bound ferulic acid was the only significant factor explaining the variation in kernel damage by larvae and in adult preference for oviposition by some grain storage insects. However, the constituent phenolic acids do not appear to be important because Bergvinson (1993) found that ferulic acid in meridic diets did not cause significant larval mortality of the ECB and that sugar conjugates of ferulic and *p*-coumaric acids extracted from resistant lines were phagostimulants.

In recent years, a laboratory bioassay based on lyophilized corn whorl tissue has been developed and refined to evaluate the leaf-feeding resistance in lines of corn to the FAW, the SWCB, and other corn insects (Williams et al., 1990, 1995; Buckley et al., 1991; Williams and Buckley, 1992; Williams and Davis, 1995). These workers concluded that growth of larvae on diets based on lyophilized leaf tissue was correlated with the average performance of the different lines.

Davis et al. (1995) studied the anatomical characteristics of corn resistant to leaf-feeding by SWCB and FAW. The most striking and consistent difference between resistant and susceptible inbreds was the thickness of the cuticle and the epidermal cell wall. The cell wall complex of resistant inbreds was 1.7× thicker than that of susceptible inbreds. This finding is consistent with our chemical data that showed resistant lines contained higher levels of fiber, including hemicellulose (Hedin et al., 1984, 1990).

This present work concentrated on the hemicelluloses that were found to be higher in resistant lines. They were extracted, purified, chemically defined, and reconstituted in diets.

METHODS AND MATERIALS

Corn Lines and Harvesting. Corn lines used in these studies were developed by the Corn Host Plant Resistance Research Unit at Mississippi State, Mississippi. They were selected for desirable resistance properties to the FAW and grown using normal agronomic practices in the area. Whorls were excised on site from plants in the midwhorl V_{8-10} (Ritchie and Hanway, 1982) stage of

growth, transported to the laboratory where they were frozen at −20°C, and dissected with minimal warming to obtain the inner green–yellow tissue that constitutes the feeding site. This section was then freeze-dried, ground in a Wiley mill (40 mesh), and the powder stored in a freezer until used in a diet, fractionated, or analyzed.

Dietary Evaluations. Larval growth on several experimental diets was compared to that of larvae fed a standard casein–wheat germ diet that had been established by Davis (1989) for the SWCB and FAW. The casein–wheat germ diet contains 17.0% casein and 14.3% wheat germ, with the remainder consisting of agar, alphacel, and other nutritional solids. Because wheat germ contains hemicellulose, it was excluded from the experimental diets and the casein was increased to 22.1% of total solids to provide an equal amount of protein. The hemicellulose and cellulose isolates were included in the diet at 15% of total solids, about half of their content in tissue. This variance was necessary because larger quantities caused diets to be too viscous for successful blending. Two diets, the first containing a mixture of 0.1% *p*-coumaric acid and 0.1% ferulic acid, and the second containing a mixture of 1.0% *p*-coumaric acid and 1.0% ferulic acid, were also included.

The treatments (diets) were arranged in a randomized complete block design with 10 replications. Each treatment was represented by four larvae fed individually in 30-ml plastic cups, each containing approximately 10 ml of diet.

The FAW and SWCB larvae were fed for 10 and 14 days, respectively, before weighing. Their rearing environment was 27.6°C, 50–60% relative humidity, with a 16L:8D photoperiod.

The data were subjected to analysis of variance (ANOVA), and the least significant difference (LSD) tests was used to compare treatment means.

Analytical Procedures. Association of Official Analytical Chemists (AOAC) methods (Horwitz, 1975) were used for the following analyses: total solids (moisture), 14.083; crude fat, 14.019; crude fiber, 14.118; acid detergent fiber, 7.056, 7.057, ash, 14.114; total protein, 2.049 (percent nitrogen × 6.25); nitrogen-free extract (NFE) by difference from 100%. AOAC methods were also used for analysis of acid detergent fiber, 973.18; and lignin (by loss on ignition), 973.18C (Helrich, 1990).

Neutral detergent fiber was determined by the methods of Van Soest and Wine (1967). From these procedures, lignin, cellulose, and hemicellulose were determined directly, and soluble cell wall contents by difference from 100%.

p-Coumaric and ferulic acids were determined by GLC-FID of their silyl derivatives, and their presence was confirmed by GLC-MS. Using the general procedures of Bergvinson (1993), freeze-dried corn whorl (5 g) was first extracted with 50% aqueous ethanol to remove chlorophyll. The residue was extracted in the dark for 4 hr with 2 N aq. NaOH (300 ml). Then the NaOH solution was extracted exhaustively with ethyl acetate. The ethyl acetate solution was dried

with Na_2SO_4 and concentrated to ca. 20 ml from which a 1-ml aliquot was silylated with *N,N*-bis(trimethyl-silyl)acetamide (Pierce Chemical Co., Rockford, Illinois). The analyses were quantitated by comparison with authentic, weighed samples of *p*-coumaric and ferulic acids (Fluka, Ronkonkoma, New York).

Extraction and Analysis of Hemicellulose and Cellulose. The procedures were based on those of Selvendran and Ryden (1990). Typically, 100 g of ground freeze-dried whorl was extracted at boiling by Soxhlet with 50% aqueous ethanol, yielding about 20% solubles including the pigments, lipids, sugars, and salts. The residue from the aqueous ethanol extraction was extracted with 1 N KOH (1:25 w/v) for 24 hr, centrifuged, and the resulting residue was resuspended in water and centrifuged a second time. The supernatants were combined and adjusted to pH 5.0 with glacial acetic acid so that the hemicellulose precipitated. The residues from the centrifugation (chiefly cellulose) and the HOAC precipitate (hemicellulose) were transferred to dialysis tubing and dialyzed with daily changes of deionized water for seven days, after which they were freeze-dried and weighted to determine yield.

Glycosyl compositions (sugars) were determined via hydrolysis with 2 mol/liter trifluoroacetic acid and subsequent preparation and analysis of their alditol acetates according to the methods of Sturgeon (1990).

Solid- and Liquid-State NMR Experiments. The ^{13}C solid-state NMR spectra of freeze-dehydrated whorl powders and their isolated hemicelluloses were recorded at 75.49 MHz on a Bruker AMX-300 spectometer with a Level I Solids Accessory using a 4-mm rotor. The Bruker pulse program cp21ev was used with a prepulse delay of 3 sec and a contact time of 3.0 msec. Each spectrum was obtained using a MAS spinning rate of 7.5 kHz and 6000 scans.

Samples of resistant (Mp 708) and susceptible (SC 229) hemicelluloses were prepared by dissolving 80 mg of each in 2.5 ml of D_2O containing 20% KOH (by volume). All spectra were referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Isotopically enriched D_2O was purchased from Isotec Inc. (Miamisburg, Ohio), and 2,2-dimethyl-2-silapentane-5-sulfonate was purchased from Cambridge Isotope Company (Woburn, Massachusetts). Both compounds were used without further purification. All NMR experiments were conducted with a Bruker AMX-300 spectrometer. The one-dimensional proton spectra were acquired at a frequency of 300.1355342 MHz using a spectral width of 6024 Hz, with 16K data points yielding a digital resolution of 0.37 Hz/point with a total acquisition time of 1.35 sec/scan. A total of 32 scans (1 sec delay between scans) were acquired and then processed with an exponential multiplication defined by a 1-Hz line broadening. The one-dimensional carbon-13 spectra were acquired at a frequency of 75.4685870 MHz using a spectral width of 19,230 Hz, with 32K data points yielding a digital resolution of 0.6 Hz/point with a total acquisition time of 3.4078 sec/scan. A total of 35,000

scans (1 sec delay between scans) was acquired and then processed with an exponential multiplication defined by a 3-Hz line broadening.

Statistical Methods. Data were combined for analysis of variance or comparison of standard errors of the mean.

RESULTS AND DISCUSSION

The results from attempts to chemically characterize the (S) and (R) whorl powders are summarized in Table 1. Crude fiber was 27% higher on average in the (R) whorls than in the (S) whorls. Proximate analyses of AB 24E (S), SC 229 (S), Mp 704 (R), and Mp 708 (R) gave results similar to those previously reported for several other (S) and (R) lines (Hedin et al., 1984, 1990). Table 1 also shows results for the contents of lignin, cellulose, hemicellulose, and cell wall solubles from determinations based on the use of AOAC neutral and acid detergent fiber procedures. Tissue from (R) plants was 20% higher on average in cellulose and 31% higher in hemicellulose, again similar to our earlier findings (Hedin et al., 1984). Lignin was very low (1.8–2.0%), and not different in (S)

TABLE 1. CONSTITUENTS OF FAW SUSCEPTIBLE AND RESISTANT CORN INNER WHORL LEAF TISSUE[a]

	Percent			
	AB 24E (S)	SC 229 (S)	Mp 704 (R)	Mp 708 (R)
Ash	8.8	6.9	8.0	8.5
Protein	25.6	26.9	17.8	18.2
Fat	1.6	1.7	1.4	1.4
Fiber	24.1	22.9	28.6	31.3
Nitrogen-free extract	40.0	41.5	44.1	40.6
Cellulose	28.0	24.6	31.8	31.1
Hemicellulose	25.5	23.8	32.9	31.7
Lignin	1.9	1.8	2.0	1.9
Cell wall contents	44.6	47.6	33.2	35.3
Isolated Hemicellulose[b]		22.3 ± 4.0		26.7 ± 3.6
Free *p*-coumaric acid[c]		0.97		0.95
Free ferulic acid[c]		1.21		1.34

[a] Association of Official Analytical Chemists (AOAC) methods (Horwitz, 1975; Helrich, 1990). Analyses in duplicate.
[b] Hemicellulose isolated on five occasions by procedures based on those of Selvedran and Ryden (1990).
[c] GLC-FID and GLC-MS analysis of silylated isolates.

and (R) whorls. The higher content of cell wall solubles in (S) whorls (35% on average) suggested higher digestibility and availability of nutrients.

To evaluate completeness of the extractions, the isolated hemicellulose and cellulose fractions were analyzed by the crude fiber procedure, which is defined as the loss on ignition of dried residue remaining after digestion of sample with boiling 1.25% H_2SO_4 and 1.25% NaOH (AOAC 14.118). As expected, the hemicellulose was solubilized (0.0–0.7%), whereas the cellulose was retained (58.8–60.4%), thus confirming that the two isolates were different and essentially free of each other; this was important information for the interpretation of subsequent laboratory bioassays.

Free *p*-coumaric and ferulic acids (as extracted with 2 N NaOH) were found to be about equal, respectively, in (S) whorls (0.95–0.97%) and in (R) whorls (1.21–1.34%) (Table 1). They were present in somewhat higher concentrations than reported by Bergvinson (1993). However, alkaline extractable *p*-coumaric and ferulic acids could not be isolated from purified hemicellulose. This is not surprising, since extraction of the hemicellulose from whorls initially had utilized aqueous NaOH, which was subsequently discarded.

To further characterize the hemicellulose, a series of isolates were prepared for determination of their substituent sugars and in sufficient quantities for dietary tests. Initially, sugar analysis via the alditol acetate procedure was performed on the untreated whorls. The recovery of neutral sugars (Table 2) (by summation) was about 21% for two (S) lines and nearly 29% for two (R) lines. Xylose and arabinose, from the hemicellulose, and glucose, chiefly from carbohydrates other than cellulose, were the major sugars. When several replications each of hemicelluloses from an (S) line (SC 229) and an (R) line (Mp 708) were analyzed for neutral sugar content, they were found to be significantly higher in the (R) line (52%) than in the (S) line (37%). However, the proportion of hemicellulosic xylose was not higher in the resistant lines. The approximate ratio of xylose to arabinose in the purified hemicellulose was 9:1, defining whorl hemicellulose as an arabinoxylan. For comparison, a corn cob arabinoxylan (isolated and analyzed by R. B. Hespell, Northern Regional Research Laboratory, USDA-ARS, Peoria, Illinois) gave a somewhat higher recovery of sugars, but the ratio of neutral sugars was fairly similar to corn whorl. A commercial xylan sample (Sigma Chemical Co., St. Louis, Missouri) yielded 64.2% neutral sugars and contained only a small amount of arabinose (Table 2).

The solid-state ^{13}C NMR analyses of the (R) and (S) whorls (Figure 1, Table 3) showed significant differences in the samples, and indicated that the (R) hemicellulose had a greater degree of cross-linking than (S) hemicellulose. The following solid-state ^{13}C NMR peaks were present in both the (R) and (S) samples: 25 (R), 55 (CH_3O), 65 (CH_2O), 75 (CH—O), 103 (anomeric C), 173 (broad carbonyl 1), and 181 (carbonyl 2) ppm. In the (R) sample, the peaks at 181 and 25 ppm were more intense than the corresponding peaks in the (S)

TABLE 2. NEUTRAL SUGARS IN FAW SUSCEPTIBLE AND RESISTANT CORN PLANT INNER WHORL TISSUES AND THEIR HEMICELLULOSE FRACTIONS

Sample[a]	Neutral sugars (%)[b]	Distribution of neutral sugars (%)[c]					
		A	X	M	Ga	Gl	Othe
Ab 24E whorl (S)	20.6	9.5	52.4	2.9	4.6	29.5	1.1
SC 229 whorl (S)	22.3	9.0	51.4	2.5	3.9	33.2	0
Mp 704 whorl (R)	29.4	9.8	61.3	1.6	3.5	23.8	0
Mp 708 whorl (R)	28.2	10.3	61.7	2.1	4.1	21.8	0
SC 229 whorl hemicellulose (S)	37.3 ± 4.3	9.5	80.8	0.3	1.4	6.7	1.4
Mp 708 whorl hemicellulose (R)	52.4 ± 5.4	8.9	82.6	0.1	0.9	6.4	1.0
NRRC corn cob xylan	75.3	15.0	76.0	0	5.0	5.0	0
Xylan, Sigma Chemical Co.	64.2	0.8	91.9	0.3	0.8	5.1	0.1

[a] Analyses performed in duplicate except for hemicellulose and cellulose. Two xylans were isolated and ana lyzed.

[b] Percent neutral sugars in sample. Differences in total from 100% largely attributable to cellulose, which resistant to acid hydrolysis, and to xylan, which on acid hydrolysis yields only about 65% of theoretic value.

[c] Sugars determined by GLC of the alditol acetates (Sturgeon, 1990). A, arabinose; X, xylose; M, mannos Ga, galactose; Gl, glucose.

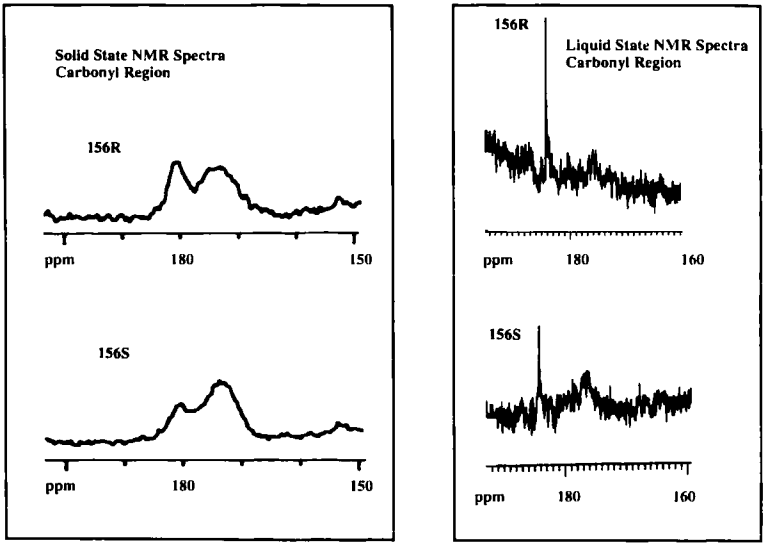

FIG. 1. Solid- and liquid-state ^{13}C NMR spectra of the carbonyl region (150–220 ppm) of SC 229 (156S) and Mp 708 (156 R) purified hemicelluloses.

TABLE 3. LIQUID- AND SOLID-STATE CARBON-13 NMR ASSIGNMENTS FOR RESISTANT (Mp 708) AND SUSCEPTIBLE CORN (SC 229) WHORL HEMICELLULOSES

Peak No.	R (ppm)[a]	S (ppm)[a]	Possible assignment
1	184.00	184.02	Carbonyl, possibly water
2	175.8 br	175.95 br	Carbonyl, possibly carboxylate
3	111.79 w	111.77 w	Possible aromatic or alkene carbon
4	104.70	104.67	α or β isomer of C-1 carbon (ketal linkage)
5	104.28	104.27	α or β isomer of C-1 carbon (ketal linkage)
6	88.80 w	88.68 w	Peaks 6, 7, 8 most likely correspond to carbons
7	82.85 w	82.86 w	contained in the furanose (five membered
8	80.95 w	80.79 w	ring) sugars
9	78.72	78.69	Most of the contributions to peak intensity in
10	76.81	76.75	this region (9-11) correspond to carbons
11	75.65	75.64	contained in the pyranose (six membered ring)
12	75.38	75.34	Sugars with some overlap with the carbons
13	65.80	65.76	contained in the furanose sugars
14	65.53	65.61	
15	25.92 w	25.92 w	Peaks 15 and 16 correspond to alkyl compounds
16	25.75 w	25.75 w	

[a]br = broad singlet; w = resonance which is less than 20% of most intense resonance.

sample, indicating that the (R) sample contained more carbonyl 2 functionalities and more aliphatic carbons than did the (S) sample. Common carbonyl compounds that appear in this region include carboxylic acids, esters, and carboxylate salts (about 5 ppm more deshielded than their corresponding acid). Since the liquid samples were analyzed in NaOH, these two carbonyl peaks can be attributed to an acid carboxyl and/or carboxylate salt because the acid would have been converted into the salt under the basic liquid NMR conditions. The liquid and solid NMR showed the same relative amounts of carbonyl 1 and carbonyl 2 compounds.

In liquid-state ^{13}C NMR studies (Figure 1, Table 3), the carbon-13 spectra of Mp 708 (R) and SC 229 (S) hemicelluloses were complicated by the relatively large molecular weights of the hemicelluloses and by the high pH of samples. The use of 20% KOH, which was necessary to dissolve the compounds, caused line broadening and baseline problems. The one-dimensional carbon-13 spectra are qualitatively very similar, as seen in Table 3, but the quantitative intensities of the carbon resonances for (R) and (S) are much different. As can be seen in Figure 1, the carbonyl resonance at approximately 184 ppm was much stronger in the (R) hemicellulose than it was in the (S) hemicellulose. However, the relative intensity of the broad carbonyl resonances centered at approximately 175.9 ppm was higher in (S) than (R). The intensity of the resonances occurring

between 90 and 80 ppm also seemed to be relatively greater in (S) than in (R). These features clearly differentiated between the two hemicelluloses.

The proton spectra (not presented) of both (R) and (S) gave only broad singlets with no observable couplings, and there were no clear differences between the proton spectra of the two compounds. ^{13}C NMR spectra were also taken on the original whorl powders. However, no differences in carbonyl carbon intensity between the (S) and (R) powders were evident, presumably because the hemicelluloses comprised only a relatively small fraction of the whorl powders.

Table 4 shows the results of five laboratory feeding bioassays, three with the FAW and two with the SWCB for comparison. In two of three bioassay tests, growth of FAW larvae on diets containing (R) hemicellulose was significantly lower than on diets containing equal amounts of the (S) hemicellulose. In the third test, larval weights on diets containing the (S) and (R) hemicelluloses were not statistically different. If it had been dietarily feasible to test the (S) and (R) hemicelluloses at higher levels, reflecting their contents in the whorl, the effect of the (R) hemicellulose may have been greater.

As with the FAW, weight gains of SWCB larvae fed the (R) hemicellulose diet tended to be lower, but not significantly so, than those fed the (S) hemicellulose diet. On the other hand, larval weights were increased significantly on diets containing the (R) cellulose.

TABLE 4. WEIGHTS OF FAW AND SWCB LARVAE FED CASEIN-BASED LABORATORY DIETS CONTAINING 15% CORN WHORL HEMICELLULOSE AND CELLULOSE (milligrams)[a]

	FAW			SWCB	
Diet[b]	Test 1	Test 2	Test 3	Test 1	Test 2
Casein-wheat germ	483.8 a			287.3 a	
Casein-wheat germ plus cellulose	484.7 a	353.6 a	226.6 bc	298.8 a	238.7 a
Casein	199.9 d			126.2 de	94.0 e
Casein plus cellulose	238.2 cd	105.2 d	175.6 c	112.4 e	
Casein plus S whorl hemicellulose	338.2 b	207.8 b	272.8 b	144.3 cd	145.5 cd
Casein plus R whorl hemicellulose	189.1 d	161.5 c	254.6 b	127.1 de	126.6 d
Casein plus S whorl cellulose	254.0 c	180.6 bc	403.7 a	147.7 c	147.4 c
Casein plus R whorl cellulose	363.9 b	176.1 c	281.3 b	201.8 b	183.1 b
LSD 0.05	54	56	21	19	18

[a]Means followed by different letters are significantly ($P = 0.05$) different according to Duncan's multiple range test.

[b]Based on the laboratory diet of Davis (1989). Hemicellulose and cellulose isolated by procedures based on those of Selvendran and Ryden (1990).

Two diets, one containing 0.1% each of *p*-coumaric and ferulic acids, and the other containing 1.0% each of these two phenolic acids added to the control diet were bioassayed in feeding tests with the FAW. The average larval weight at 10 days on diets containing 0.1% each of the phenolic acids was 184.9 mg. At the 1.0% level, the average weight was 127.6 mg. The lower level of this phenolic acid mixture was approximately that which was found by Bergvinson (1993), and the higher level reflected that found by us (Table 1).

Freeze-dried whorl powders have been dispersed in laboratory diets to evaluate FAW resistance (Williams and Buckley, 1992; Williams et al., 1995). The aqueous residue from resistant whorls was associated with poor growth (Williams and Davis, 1995). However, to identify the actual basis of resistance, it is necessary to devise extractive procedures that conserve biological activity upon recombination of fractions. Unfortunately, no entirely satisfactory procedures could be established because extraction at reflux with presumably mild solvents, including water, ethanol, and 50% aqueous ethanol, failed to give extracts that elicited growth upon recombination with the residue. An all-purpose vitamin mixture partially restored activity, but this approach could not be optimized. Aqueous extractions at intermediate temperatures (65–70°C) gave full activity upon recombination with the residue. However, further treatments of the extracts or residues resulted in lost activity, so efforts to use a bioassay based on conserving the activity of whorl powder were abandoned. An alternative strategy was then adopted to isolate fractions or components that differed in concentration in (S) and (R) lines, and then to evaluate their biological activities in laboratory diets.

Hartley et al. (1988) proposed that a portion of the phenolic acids may become bound to hemicellulose through a mechanism in which a four-membered ring is formed from the double bonds of the side chains in either a head-to-tail fashion to form truxillic acids, or in a head-to-head fashion to form truxinic acids. Both phenolics may be attached to arabinose and xylose prior to the phytochemical reaction. The cyclobutane dimers consist of both ferulic and *p*-coumaric acid, and it has been hypothesized that they may constitute a defense against insect attack (Hartley and Ford, 1989; Iiyama et al., 1990; Bergvinson et al., 1994). The levels of these cyclobutane dimers are highest in tissues that intercept sunlight, with leaf blades having the highest levels, followed by sheath and stem tissue (Eraso and Hartley, 1990). Head-to-tail dimers are generally in the greatest abundance (Morrison et al., 1991). The formation of these dimers in full sunlight may explain why maize plants grown in greenhouses are more susceptible to the ECB feeding on leaves, as reported by Guthrie et al. (1986) and Bergvinson et al. (1994).

In summary, more hemicellulose was found in (R) whorls than in (S) whorls. Using standard analytical procedures, (R) lines were also found to contain more crude fiber and cellulose, but less protein and cell wall solubles. NMR

evidence was obtained for a higher level of carbonyl carbon in (R) hemicellulose. In dietary tests, larval weight gains were higher on (S) hemicellulose incorporated in diets at the same level as (R) hemicellulose. These data suggest that the lower weight gains can be attributed to the relative amounts and differences in structure of hemicelluloses.

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INTRA- AND INTERINDIVIDUAL VARIATION IN FLANK GLAND SECRETIONS OF FREE-RANGING SHREWS *Crocidura russula*

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Abstract—Individual differences in flank gland secretions were examined among males of the monogamous shrew *Crocidura russula* during the breeding and nonbreeding seasons. Gas chromatography was used to measure intra- and interindividual variation of flank gland secretions of free-ranging shrews from different populations. The number of compounds detected by gas chromatographic analyses was correlated with body mass, flank gland size, and the presence of blood parasites in individual shrews. Very few compounds were detected from the flank gland area of juvenile males. After they reached sexual maturity, however, the number of compounds detected from the flank gland secretions increased significantly. At the beginning of the reproductive season 48 different compounds were detected from male flank gland secretions. In the middle of the breeding season 70 compounds were detected, while only 11 compounds were detected during the nonbreeding season. Few compounds were common to all males. There were more volatile compounds in the flank gland secretions of males in the beginning of the breeding season than later in the breeding season. Males from the same population had fewer differences in the elution profile of compounds than males from different populations indicating that individuals from a distinct population have similar elution profiles of compounds and that each population has its own type of elution profile. No correlations were found between the number of compounds

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detected by gas chromatography for each male and the male's body mass or flank gland size. Blood parasites (trypanosomes, *Trypanosoma crocidurae*) were found in only three of 30 males investigated.

Key Words—*Crocidura russula*, flank gland secretions, gas chromatography, flank gland size, body mass, blood parasites, shrews.

INTRODUCTION

Social communication through the use of odors in small mammals is well known (Eisenberg and Kleiman, 1972; Mykytowycz and Goodrich, 1974; Stoddart, 1976; Brown and Macdonald, 1985). In rodents, the scent glands and their odors play an important role in reproduction, mate selection, parent–offspring interactions, interindividual recognition, and scent marking of territory (see review in Brown, 1985a,b). The secretions of scent glands can be studied with chemical analyses for species, sex, and individual differences (Albone, 1984). Few studies have been carried out on the role of olfaction in the social behavior of shrews and other insectivores, probably because they are difficult to observe in their natural habitat and they are rarely used in the laboratory.

Shrews (Soricidae) have poor visual acuity and are mainly nocturnal, thus olfaction may play a major role in their social communication (Poduschka, 1977; von Holst, 1985; Poduschka and Wemmer, 1986). This conclusion is based on the facts that shrews have a well-developed olfactory system (Sigmund and Sedlacek, 1985) with a prominant vomeronasal organ (Ärnbäck-Christie-Linde, 1914) and numerous cutaneous glands (Niethammer, 1962; Murariu, 1971, 1972, 1973). The largest odoriferous glands are the lateral flank glands present in most shrews of both sexes (Geoffroy-St-Hilaire, 1815). Histological (Johnsen, 1914; Eadie, 1938; Pearson, 1946; Murariu, 1973; Balakrishnan et al., 1986) and physiological studies (Pearson, 1946) especially with castrated shrews (Dryden and Conaway, 1967; Balakrishnan and Alexander, 1976), have shown that the flank glands of shrews are under hormonal control and are generally more developed in males than in females, especially during the breeding season. Cantoni and Rivier (1992) showed a sexual dimorphism in flank gland secretions, with males having numerous compounds in their flank gland secretions revealed by gas chromatography analysis while no compounds were detected from the flank gland area of the females.

Scent-marking behaviors in mammals are very common and provide the means by which social odors can be transmitted over distance and time or directly to conspecifics by partner marking. In shrews the flank gland secretions may be useful during the reproductive season for finding a mate, and partner marking may promote the sexual receptivity of the female by the male (von Holst, 1985). *Suncus murinus* and *Blarina brevicauda* scent mark by rubbing

their flank glands against objects (Tennant et al., 1987; Platt, 1976), and these flank gland marks may be used for territorial defence (Pearson, 1946; Poduschka and Wemmer, 1986).

In shrews (*Suncus murinus* and *Blarina brevicauda*), it has been shown that the postauricular glands, ventral glands, and lateral flank glands are associated with reproduction, with males exposing their postauricular glands to female biting, which promotes the receptivity of the female (Poduschka and Wemmer, 1986). Scent marks of males may also be used for mate selection and mate recognition, as has been shown in rodents (Newman and Tang Halpin, 1988; Tang-Martinez et al., 1993; Johnston and Jernigan, 1994). Cantoni and Rivier (1992) found that few compounds detected by gas chromatography were common to different individual male *C. russula*. This suggests that each male *C. russula* produces a unique odor that could play an important role in mate choice and mate recognition in this monogamous species.

The aim of the present study was to use gas chromatography to analyze individual differences in flank gland secretions among male *C. russula* by measuring intra- and interindividual variations of flank gland secretions of free-ranging shrews coming from different populations during the breeding and nonbreeding season. The number of compounds detected by gas chromatography analysis of the flank gland secretions were correlated with morphological measures (body mass and flank gland size). Blood samples were analyzed for the presence of blood parasites, because the presence of these parasites could affect the physiology of the animal (Sebek, 1978; Milinski and Bakker, 1990; Harvey and Bradbury, 1991). Parasitized male mice are less attractive as mates than healthy males (Kavaliers and Colwell, 1995). These particular parameters were assessed because of their potential role in determining the attractiveness of a male to a potential mate (Cockburn, 1991; Milinski and Parker, 1991; Harvey and Bradbury, 1991).

METHODS AND MATERIALS

Study Animals. Crocidura russula, originally from North Africa, are found in Switzerland only near human habitation. The breeding season of *C. russula*, which is a monogamous shrew (Ricci and Vogel, 1984; Cantoni and Vogel, 1989), is strongly influenced by climatic conditions and altitude (Genoud, 1981). The shrews in this study were all trapped in residential gardens in the neighborhood of Lausanne (altitude 500 m, Vaud, Switzerland). In this region the breeding season generally begins in March and ends in September. *C. russula* have a life-span of 12–18 months (Vogel, 1972) and females have an average of three or four litters per year with a mean of 4.4 pups per litter (Besançon, 1984). At weaning, the juveniles weigh 6–9 g (Besançon, 1984). They grow

rapidly and may become reproductive adults within 42 days of birth when population densities are low (Genoud, 1981; Besançon, 1984). Early the following spring, the individuals that have survived are reproductively active and weigh 10–13 g. In sexually active males and only during the breeding season, the lateral flank glands become enlarged and produce strong odoriferous secretions (Besançon, 1984; Cantoni, 1990). In females, sexual maturity is characterized by evidence of gestation. There is no increase in size or secretion rate of the flank gland in sexually active females (Murariu, 1973; Cantoni and Rivier, 1992).

Collection of Flank Gland Secretions. During the breeding season in 1988 and 1989, 27 adult male, 12 juvenile male, and 17 subadult male *C. russula* were trapped in two gardens in Lausanne. All individuals were trapped in Longworth traps (Penlon, Abingdon, UK). The flank gland secretions of the males were collected as soon as the males were removed from the traps. This was done to prevent the stress of being handled from influencing the scent gland secretions and also to prevent any contamination of the gland areas from our hands. The method used to collect the flank gland secretions was similar to that used by Erlinge et al. (1982) as modified by Cantoni and Rivier (1992). This method consisted of rubbing each flank gland back and forth four times with a piece of filter paper (Whatman 40, 3 × 3 mm) held with tweezers, which were cleaned with 70% alcohol between each individual sample. Two samples were taken from each flank gland and then the four filter papers collected from each individual were extracted and stored in 200 μl dichloromethane ($CHCl_2$) inside a hermetically sealed vial and kept at −30°C until they were analyzed by gas chromatography. When no flank glands were visible, their position was estimating using the method described by Murariu (1973) and the area rubbed with filter paper in the manner described above. After flank gland samples were taken, the shrews were identified, weighed, sexed, marked by toe-clipping and released at the point of capture.

Control samples were regularly taken at the beginning of each new trapping session and consisted of four pieces of clean filter paper dropped directly into 200 μl $CHCl_2$ and stored at −30°C. This was done in order to check for any contamination of the solvent ($CHCl_2$) and/or the filter papers during the study period.

In December 1988, during the nonbreeding season, two old adult males (born in 1987) and nine young adult males (born in the current year) were trapped in the same two gardens and their flank gland secretions were collected using the method described above.

During the breeding season in 1993, 33 adult males were repeatedly trapped in six different gardens. These gardens were far apart (2–20 km) and separated by roads, fences, and other barriers, such that migration of shrews between

these gardens was unlikely. One or two trapping sessions were done in each of the six gardens. Each trapping session lasted 12–48 hr, and each male was trapped one to six times. The flank gland secretions of these 33 males were collected as described previously, and the size of each of the two flank glands from 30 of the 33 males was measured with vernier calipers each time the male was trapped. The shape of the gland is rather oval and the length was taken at the longest point of the gland. The means of all measures of gland length for each male were used for analysis. Blood samples were taken the first time each of the 30 males were trapped. Blood was collected when the individuals were marked by toe clipping or from a small transverse cut at the base of the tail made with a sterilized scalpel and the blood was smeared on glass slides (two slides per male). Finally, each male was weighed. Because traps were baited with meal worms (*Tenebrio molitor*), however, some individuals had a full stomach and some did not, depending on how long they had been in the trap. To avoid this bias, the 30 males were kept in individual cages for 2 hr after their capture without food but with water, and weighed again. The second weight was used for analysis. Males were then released at their point of capture.

Gas Chromatographic Analysis of Flank Gland Secretions. Gas chromatographic analysis of the flank gland secretions was performed according to the procedure of Rivier and Baumann (1976) as modified by Cantoni and Rivier (1992). The vials containing the four filter paper wipes from each animal in 200 μl $CHCl_2$ were each considered as an individual sample. To each sample, 10 μl of standard mixture of the *n*-alkanes [solution of 0.033% (w/w), $C_{14}H_{30}$, $C_{15}H_{32}$, and $C_{16}H_{34}$ in hexane] was added as an internal standard. This solution was chosen because its elution time is very short and does not interfere with the elution time of the compounds contained in the samples. From each sample, 1 μl of solution was injected (250°C) into a gas chromatograph (Hewlett Packard 5790) capillary column (25 m × 0.2 mm) containing a methyl silicone phase (OV-101). The oven temperature was initially at 130°C, held there for 1 min, and then increased 10°C/min to 280°C, and maintained for 10 min (12 PS, pressure at the head of the column). These eluting compounds were detected by a flame ionization detector maintained at 300°C.

Peak retention times and peak areas were measured. The same elution program was used for all analyses, thereby permitting comparison of the number of peaks, area of peak, and peak retention time of the detected substances of the different chromatograms. Peak retention times were rounded up to the first decimal and, when necessary, peak retention time was adjusted as a function of the mean elution time of the three peaks of the internal standard solution. The three peaks (C_{14}, C_{15} and C_{16}) of the internal standard were used to evaluate any minor shifts of the retention time that may occur from one chromatographic analysis to another even if the same analytical conditions are used. Therefore,

these three peaks were useful for the comparative study of the chromatograms in order to identify homologous peaks. No identification of the different compounds detected was undertaken.

Reliability of Flank Gland Secretion Collecting Method. In order to test the reliability of the method for collecting the flank gland secretions in the field, four to six samples of flank gland secretions were taken over a short period of time (from 3 hr to 7 days), from each of four males trapped in the same garden. All samples were analyzed by gas chromatography as described above. The aim of this experiment was to determine whether our method of collecting the flank gland secretions gave repeatable results for each male. In order to test whether the gas chromatograms from repeated samples from the same individuals resulted in the same set of compounds being detected, we used a Mantel test (Mantel, 1967), which is a general method of matrix comparison dealing with two similarity or distance matrices obtained independently and describing the relationships among the same objects. The similarity or distance matrices can be obtained using an appropriate association coefficient from Legendre and Legendre (1979). In our case, the first matrix contained Euclidean distance data, r_{Huben} (Legendre and Legendre, 1979), calculated on the presence or absence of the compounds detected by gas chromatography within the flank gland secretions of the same individual and/or different individuals.

The second matrix was the theoretical matrix that corresponded to our hypothesis that variability in eluting compounds within the same individual is smaller than variability in eluting compounds between different individuals. This matrix had a maximal distance for the comparison of the secretions from two different individuals and a null distance for the comparison of secretions from the same individual. The observed data in the first matrix can then be compared to the second matrix, which represents the data expected. The null hypothesis is that the first matrix is not correlated with the second matrix.

In Mantel's (1967) procedure, correlations are calculated between pairs of matrices and tested by permuting at random the rows and corresponding columns of one matrix 1000 times. After each permutation, the correlation statistic is obtained, producing a set of values of the normalized Mantel statistic, r, obtained under H_0. These values represent the sampling distribution, which is used for the significance test. The "R Package for Multivariate Data Analysis" (P. Legendre and A. Vaudor, University of Montreal) was used for this analysis.

Statistical Analysis of Gas Chromatograms. Compounds in the flank gland secretions of the different males, detected by gas chromatography, were analyzed with correspondence factorial analysis, which is a multivariate analysis similar to principal component analysis and developed by Benzecri (1973). In a principal component analysis, the factors (first, second, . . . , etc. principal components) are the combination of the variables and allow one to project the objects in the space of the variables. In the correspondence factorial analysis the factors are

the linear combination of the variables and the objects and, therefore, allow one to project the objects and the variables together in the same space. In our case, the individuals were considered as the objects and the areas of the detected compounds (peaks), according to their retention time, were considered as the variables. Principal component analysis was used by Natynczuk and Macdonald (1994) to analyze rat haunch odors. By using a correspondence factorial analysis instead of a principal component analysis, we were able to measure and visualize the relationships between the individuals, the peaks, and the individuals and the peaks.

Variability in compounds detected from the flank gland secretions of individuals coming from the same population (same garden) and individuals coming from different populations (separate locations) were tested with the Mantel test using the procedure described above. The first matrix contained Euclidean distance data, r_{Huberl} (Legendre and Legendre, 1979), calculated on the presence or absence of the compounds detected by gas chromatography on the flank gland secretions of individuals from the same or different populations. The second matrix had a maximal distance for comparison of the secretions of individuals from different populations and a null distance for comparison of individuals from the same population. The null hypothesis, H_0, is that the first matrix is not correlated with the second matrix.

Pearson correlations were performed between the total number of detected compounds, body mass, and flank gland size.

Blood Smear Analysis. The blood samples were analyzed for the presence of parasites such as *Plasmodium* or *Trypanosoma*. To perform this analysis, the blood smears on each slide were colored with Giemsa (Kit Colorrapid, Bioreac, Lausanne, Switzerland), and the slides were examined under a microscope (Leitz Laborlux D, magnification 500×) for the presence of parasites.

RESULTS

As a preliminary test of the reliability of the sampling technique, four males were tested and we found that the variability in the compounds of secretions from different individuals (Mantel test: $r_{\text{Huberl}} = 0.516$, $P < 0.05$). This means that all of the eluting compounds detected in the flank gland secretions of one individual are characteristic of that individual and the sampling method used is reproducible. Thus, our sampling of the flank gland secretions is accurate enough to distinguish different males and, therefore, only one sample of the flank gland secretion of each male was collected.

The flank gland secretions of 60 different adult males trapped in the breeding seasons of 1988, 1989, and 1993 were analyzed by gas chromatography. A mean of 54.6 ± 21 peaks were detected per adult male and up to 215 different

compounds were found over all males (Figure 1A). Each male had its own elution profile of compounds (combination of peak retention times and peak areas), and only a few peaks per male were common to all males (see Cantoni and Rivier, 1992). The elution profile of the peaks for the same male were similar in samples taken within a short period of time (several hours to seven days) (compare Figure 2B and C), but the elution profile of peaks for the same

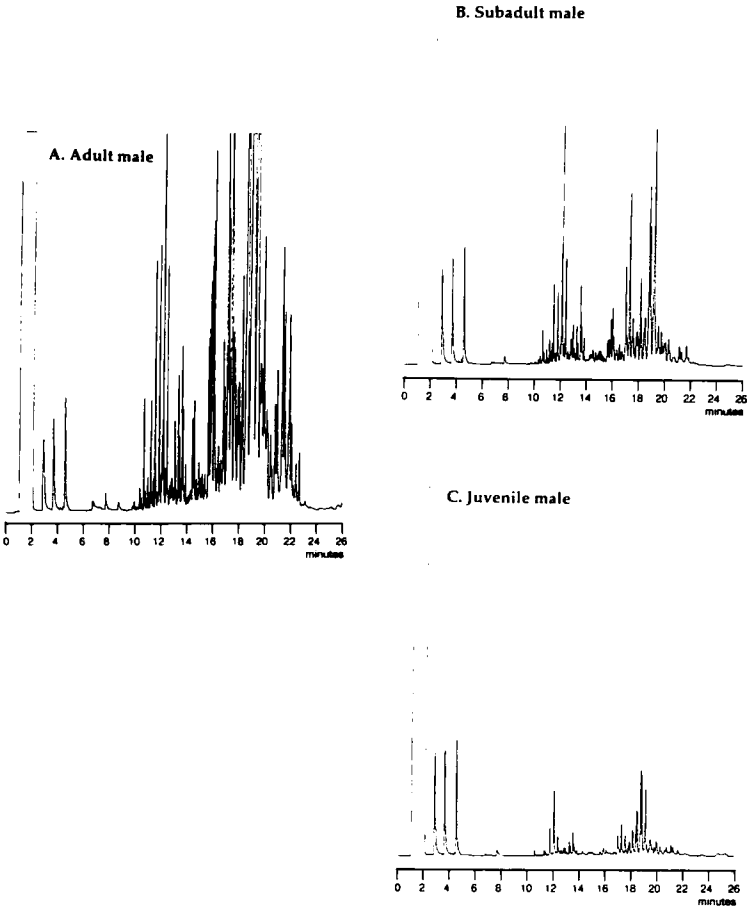

FIG. 1. Typical gas chromatograms obtained for the flank gland secretions of male adult, subadult, and postweaning juvenile *C. russula*. The first peak of each trace is the solvent front and the three following peaks are the internal standard peaks ($C_{14}H_{30}$, $C_{15}H_{32}$, and $C_{16}H_{34}$).

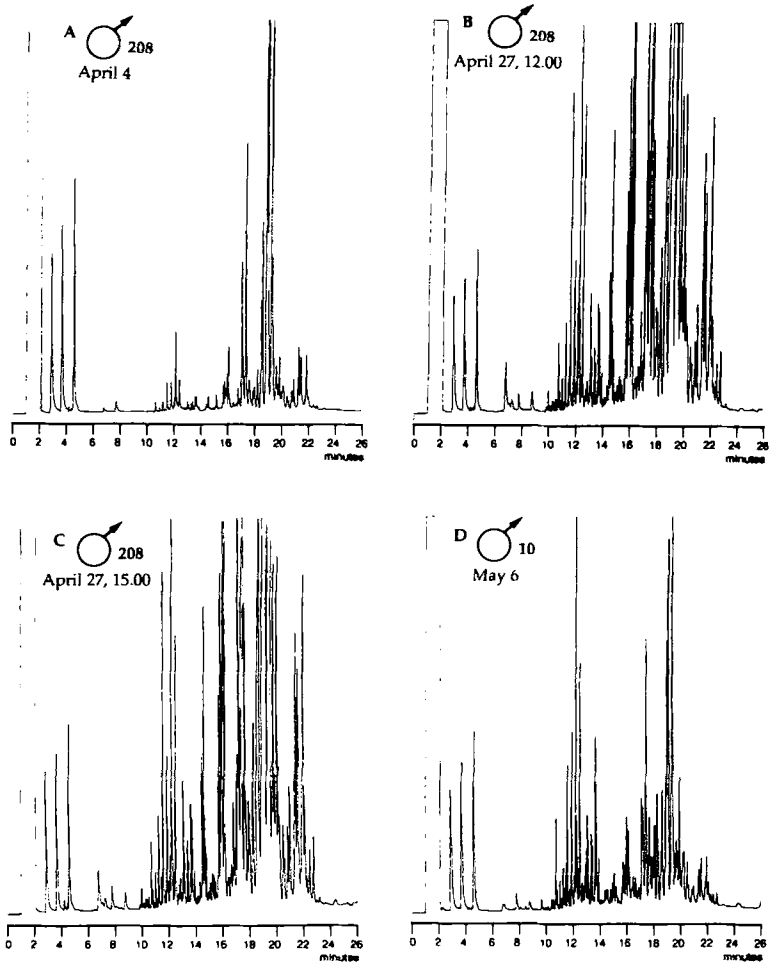

FIG. 2. Examples of gas chromatograms obtained for the flank gland secretions of one adult male (number 208) sampled on (A) April 4, 1993, (B) April 27, 1993 at 12:00 hr, and (C) April 27, 1993 at 15:00 hr. The gas chromatogram obtained from the flank gland secretion of another adult male (number 10) from another garden and sampled on May 6, 1993, is shown in (D). The abscissa represents the elution time (minutes) and the ordinate indicates the detector response. The first peak of each trace is the solvent front and the three following peaks are the internal standard peaks ($C_{14}H_{30}$, $C_{15}H_{32}$, and $C_{16}H_{34}$).

male changed over the breeding season (compare Figure 2A and C). The area of each peak indicates the amount of each chemical in the flank gland secretion. Table 1 shows the retention times for the five largest peaks in each adult male at four sampling periods. Most individuals had a different set of five largest peaks, and only a few of these peaks were common to all individuals in the same sampling period. Therefore, Table 1 indicates many individual differences in the chemicals in the flank gland secretions.

Chromatograms obtained from the flank glands of 17 subadult males trapped in 1988 showed a mean of 52.1 $\pm$ 12 peaks per male (Figure 1B). In general, the peak areas of the compounds detected for these subadult males were smaller than those detected for the adult males (compare Figure 1A and B). These subadults were young males that had yet not reproduced but were almost sexually mature according to Besançon (1984).

The chromatograms of the flank gland secretions of the 12 postweaning juveniles trapped during the breeding season in 1988 showed few compounds (Figure 1C), with a mean of 7.5 $\pm$ 4 peaks per juvenile.

The number of different compounds detected in the flank gland secretions of the adult males changed over the reproductive cycle. At the beginning of the breeding season, in April and May, adult males had, respectively, 48.1 $\pm$ 16 ($N = 27$) and 56.9 $\pm$ 25 ($N = 26$) peaks, and there was a significant increase in the number of peaks between these two months (Wilcoxon signed ranks test: $Z = 1.965$, $P = 0.049$). The number of peaks increased from May until the middle of the breeding season in July (71.0 $\pm$ 8, $N = 7$) peaks but not significantly (Wilcoxon signed ranks test: $Z = -1.572$, $P = 0.116$). During the nonbreeding period (December) only 13.1 $\pm$ 15 ($N = 11$) peaks were observed, which was significantly fewer peaks than in the breeding season (Wilcoxon signed ranks test: from April to December $Z = -2.937$, $P = 0.003$; from May to December $Z = -2.936$, $P = 0.003$; and from July to December $Z = -2.366$, $P = 0.018$). In December the flank gland size decreased, and in most individuals the glands were regressed and not visible. In December the sample was composed of nine young adult males (born in the current year) and two older adult males (born the previous year). Even though the two older males had a longer breeding season than the young ones, they were all in a nonreproductive state.

In 1988, several males were trapped repeatedly during the breeding and nonbreeding seasons, and the variation in the flank gland compounds detected by gas chromatography was analyzed using correspondence factorial analysis (Benzecri, 1973). This analysis allowed us to describe and to visualize associations or separations between variables (peak retention times and peak areas), between objects (individuals), and between variables and objects. These associations or separations are plotted as a function of the three main factors explaining the total variance of the analysis. For the sake of clarity, the results of the analysis were plotted into two figures having the same spatial configuration.

TABLE 1. RETENTION TIMES (RT) OF DIFFERENT COMPOUNDS (PEAKS) FOUND AMONG FIVE LARGEST PEAKS (AREA) OBSERVED IN EACH ADULT MALE AS FUNCTION OF SAMPLING PERIOD IN 1988 AND 1989.[a]

Sampling period (month, year)	Retention times of the five largest peaks																	Individuals (*N*)
April 1988, 89																		
RT	9.8	10.7	11.0	11.3	11.6	16.1	16.2	16.4	17.0	17.5	17.7	17.8	17.9	18.1	18.7	19.0	20.6	
N	1	5	7	13	5	2	1	10	2	5	1	1	2	12	1	1	1	14
May 1988, 89																		
RT	7.8	10.9	11.8	12.1	12.4	12.7	13.9	17.6	17.9	18.9	19.1	19.6	20.0	20.5				
N	1	1	2	9	13	6	1	2	10	1	3	1	2	13				13
July 1988																		
RT	10.7	11.9	12.2	12.5	13.6	17.6	18.6	19.0	19.5	19.9	20.9							
N	1	3	7	1	5	4	3	1	2	7	1							7
December 1988																		
RT	7.8	6.9	9.1	12.0	12.3	13.5	13.8	15.3	19.3	20.2	21.3	22.1						
N	9	8	1	1	3	2	2	1	3	4	2	2						11

[a]The number (*N*) below the retention time indicates in how many individuals the peak was found among the five largest peaks at this period of the year. In December 1988, some individuals had less than five peaks, therefore the sum of the numbers (*N*) below the retention times are not equal to five times the number of individuals (11 in December 1988).

Figure 3 represents the individuals (objects) and Figure 4 the peaks (variables). Furthermore, peaks (Figure 4) were grouped and represented (on the figure only, not in the analysis) with three different symbols according to their elution time. The individuals (Figure 3) and the peaks (Figure 4) were both projected on the planes formed by the first three factors, explaining, respectively, 15%, 10%, and 9% of the total variance of the analysis.

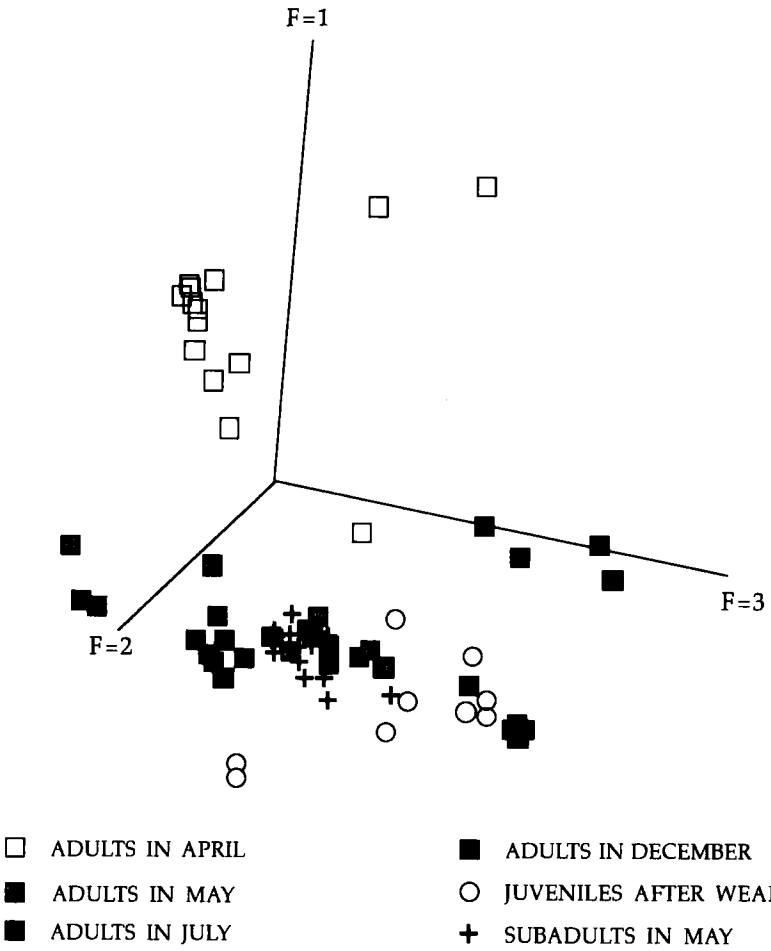

FIG. 3. Projection of the individuals (objects) in the planes formed by the first three factors (F1, F2, and F3) of the correspondence factorial analysis of the flank gland secretions of male *C. russula* during the breeding and nonbreeding season.

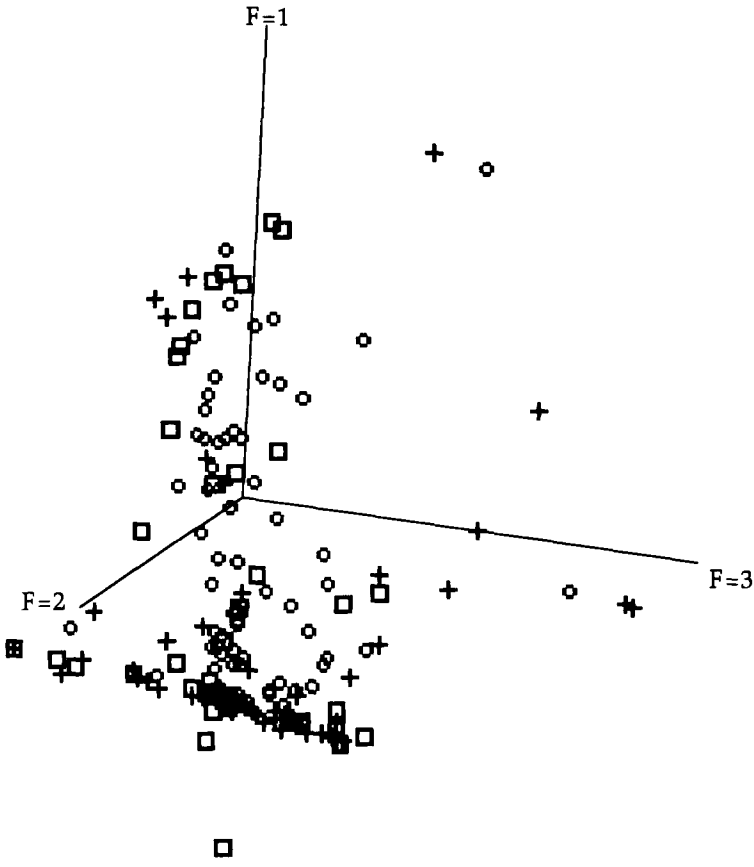

FIG. 4. Projection of the peaks (variables) in the planes formed by the first three factors (F1, F2, and F3) of the correspondence factorial analysis of the flank gland secretions of male *C. russula* during the breeding and nonbreeding season.

This analysis showed that individuals trapped during the same month are grouped together (Figure 3), indicating that they have a similar elution profile of compounds even if they do not have identical peaks. This analysis also showed that individuals trapped during a particular time of the year do not have the same elution profile of compounds as they do when trapped during another

time of the year (adults from April to July as compared to adults in December). This indicates that the flank gland secretions of each individual changed during the year. The postweaning juveniles are grouped between the subadults and the adults trapped in December and May (Figure 3). This is principally due to the fact that juveniles have few compounds detected by gas chromatography, which is also the case for individuals in December. The juveniles also have an elution profile of compounds that is quite similar to that of the individuals trapped in May, a period of the year when these juveniles were also trapped. Subadults and adults trapped in May were grouped together even if adults had generally more compounds than did subadults. Several peaks are linked with certain individuals or groups of individuals (compare Figures 3 and 4), indicating that these compounds are characteristic of these individuals. Associated with the individuals trapped at the beginning of the breeding season (adults trapped in April), we find more volatile compounds (compounds eluting between 2.7 and 17.9 min) than later in the breeding season (adults trapped in May and July), when heavier compounds were detected (Figure 4).

The flank gland secretions of the 33 males from six different populations collected in 1993 were analyzed to detect within- and between-population variability in flank gland secretions. A mean of 5.5 $\pm$ 1.8 individuals per population were sampled. The variability in the compounds detected between individuals and between populations was tested with the Mantel (1967) test. The results showed that the differences in the elution profile of compounds detected are significantly larger between populations than within populations (Mantel test: $r_{\text{Huber}} = 0.499$, $P = 0.04$), indicating that individuals from a distinct population have similar elution profiles of compounds and that each population has its own elution profile (Figure 2A, B, vs. D).

In order to test a possible correlation among the number of peaks, flank gland size, and potential male quality expressed by body mass and by absence of blood parasites, 30 adult males trapped in April and May 1993 were analyzed. There was a mean of 41 (SD = 15) peaks detected by gas chromatography from the flank secretions of these males. These males had an average flank gland length of 9.1 $\pm$ 0.8 mm, and an average body mass of 12.6 $\pm$ 1.2 g. The number of compounds detected in the flank gland secretions of the 30 males was not correlated with body mass ($r = 0.046$, $P = 0.807$) or flank gland size ($r = 0.164$, $P = 0.387$). However, body mass and flank gland size were correlated ($r = 0.391$, $P = 0.033$). These results indicate that the variance in the number of compounds detected between individuals depends on factors other than body mass and flank gland size. Blood parasites (trypanosomes, *Trypanosoma crocidurae*) (Brumpt, 1923) were found in only three males, two of which came from the same population. Due to the existence of blood parasites in only three individuals, no correlation was performed between the number of peaks detected and this physiological parameter.

DISCUSSION

Our results show that the flank gland secretions of male *C. russula* vary with the age and reproductive state of the male. These changes occur over four steps and involve the number and the amount (peak area) of compounds as well as the retention time of the compounds detected. As males grow from juveniles to subadult males, there is a large increase in the number of compounds detected in flank gland secretions (from 7 to 52) and a small change in peak area, as indicated by the height of each peak in Figure 1. During the change from subadult to reproductive adult, there is a small increase in the number of compounds detected (from 52 to 55), and a great increase in the amount (peak area) of the compounds detected (see Figure 1). Thirdly, during the breeding season, there are changes in the elution profile of compounds. At the start of the breeding season, there were more volatile compounds detected than later in the breeding season, where more heavier compounds were detected. Finally, in the nonbreeding season, there was a drop in the number and area of the compounds detected. The juveniles born late in the breeding season generally reached the subadult stage at the end of the breeding season and produced few or no flank gland secretions. During the nonbreeding season the population consisted principally of young adults, subadults, and few older adults, all of which can be considered as sexually mature but in a nonreproductive state. Indeed, Besançon (1984) observed a few cases of reproductive behavior during the nonbreeding season in an especially mild winter. Few compounds were detected from the flank gland secretions collected during the nonbreeding season, and these were similar for all males.

The age-related changes in flank gland secretions are probably related to the increased androgen secretions as these males reached sexual maturity. Indeed, Dryden and Conaway (1967) and Balakrishnan and Alexander (1976) have shown that the flank gland secretions of *Suncus murinus* were under gonadal hormonal control. They demonstrated that castration greatly reduced the production of odoriferous secretions and scent-marking behavior in this species. When these authors gave gonadal hormones in food pellets to castrated *S. murinus*, there was a reactivation of the flank gland secretions and scent marking behavior. At the onset of the reproductive season of *C. russula*, testes mature rapidly (Besançon, 1984), increasing androgen levels, which might be the cause of the development of flank gland secretions characterized by the large increase in detected compounds. The same phenomenon occurs for the postweaning juveniles, born early during the breeding season, which become adults in the same breeding season. Gonadal hormones are also likely to influence the decrease of flank gland secretions, especially when shrews enter their nonbreeding period. Nonbreeding is characterized by a strong regression of the flank glands, which produced no odorous secretions.

Our results also show that differences in the compounds detected in the flank gland secretions of males during the breeding season are significantly smaller among males coming from the same population than they are among males coming from different populations. This finding raises the question of whether the flank gland secretions are related to genetic factors. Favre and Vogel (1992) used DNA fingerprinting to show that populations of *C. russula* are highly inbred. Furthermore, it was noted that breeding dispersal is biased towards females for this species (Favre and Vogel, unpublished data). These different points suggest that the chance of trapping related males (i.e., brothers or cousins) is fairly high within a defined population. This factor could have influenced our results. Nevertheless, the notion that the elution profile of compounds detected in the flank glands of male *C. russula* is genetically based is yet to be shown conclusively. Further experiments involving comparative gas chromatographic analyses of flank gland secretions of related and unrelated individuals need to be performed.

External factors can influence the elution profile of compounds detected in flank gland secretions. Changes in diet can significantly alter the odors from skin glands, urine, and feces (Beauchamp, 1976; Skeen and Thiessen 1977; Schellinck et al., 1992, Brown and Schellinck, 1992, Salamon, 1996). This could explain a part of the change in the flank gland secretions of *C. russula* occurring during the breeding season, which may be correlated with seasonal changes in prey species leading to changes in the shrew's diet. The fact that we have found significantly fewer differences in the compounds detected among males coming from the same population versus males coming from different populations may be due to local variations in invertebrate diversity or other environmental factors that could influence the elution profile of compounds detected.

Bacteria are another external factor that may affect the production of secretions by scent glands (Brown, 1979; Albone, 1984). The external region of the flank glands in shrews is probably colonized by diverse bacteria. During the nonbreeding period, several male and female *C. russula* share a communal nest and rest together in very close contact (Cantoni and Vogel, 1989). During this period, which lasts until the onset of the breeding season, the bacterial fauna of different individuals can be easily mixed. As only a few communal nests are observed per population, the hypothesized existence of local common bacterial fauna could influence the elution profile of compounds detected within a population by gas chromatography and therefore lead to a higher similarity within populations.

We found no correlations among body mass, flank gland size, and the number of peaks detected by gas chromatography in the flank gland secretions of *C. russula*. This indicates that the size of the flank glands and body mass have no direct influence on the number of compounds detected. However, in this analysis we did not take into account the type (retention time) of peaks

detected; only the number of peaks was considered. Perhaps only a few compounds are playing a direct role in the social behavior of shrews. We can not even be sure that the strong musky scent perceived by humans is the main odor component detected by shrews. Further studies of flank gland secretions should not only identify all the compounds by gas chromatography–mass spectrum analysis, but also test them in bioassays in order to understand their function.

We found only three individuals among the 30 we examined that were contaminated with blood parasites. The *Trypanosoma* parasite found has already been described for *C. russula* (Edrissian et al., 1976; Walter and Liebisch, 1980) and for other species of shrew (Sebek, 1978). These three individuals had normal body mass with no apparent signs of poor health. Furthermore, all three were paired with a female. Very little is known regarding the effects of this blood parasite in small mammals. Helminthes, which often heavily infest shrews (Vaucher, 1971), may have a stronger influence on male body conditions.

Our findings show that male *C. russula* produce individually distinct flank gland secretions that undergo several changes during their life-span. Many complex factors are probably involved in the initiation and regulation of these secretions. Several hypotheses on the function of scent glands in shrews have been suggested. Johnsen (1914) and Eadie (1938) suggested that they function as a predator repellent. But why do females, which have the same predators as males, have no active glands? Many shrew species are solitary, thus scent marking of territories was suggested (Pearson, 1946). For the highly territorial *Sorex*, we would expect to find scent gland activity during winter when territoriality is at its maximum (Croin Michielsen, 1966). *Sorex* male flank glands, however, develop only in spring. One of the oldest interpretations of flank gland function (Geoffroy-Saint-Hillaire, 1815) deals with mate localization and male recognition. In the monogamous *C. russula*, females are able to discriminate between the odors produced by the flank glands of their partner and those of other males (Cantoni et al., unpublished data). Finally, according to Rissman (1987, 1989), female sexual receptivity in *Suncus murinus* is enhanced by male-related chemical cues. The different hypotheses are probably all parts of the reality, but flank glands are not the unique source of pheromones in shrews. Other glands, such as the postauricular glands (Dryden and Conaway, 1967), anal and subcaudal glands (Niethammer, 1962), and the ventral gland in *Cryptotis* (Pearson, 1946) certainly contribute, along with urine and feces, to a complex system of chemical signals. Moreover, among Soricidae, there are important differences in social behavior (Cantoni, 1993), reproductive strategies (Genoud and Vogel, 1990), and energetic strategies (Vogel, 1980, Genoud, 1988), suggesting the existence of different roles played by flank gland secretions in different species of shrew.

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VOLATILE COMPOUNDS FROM INTERDIGITAL GLAND OF MALE WHITE-TAILED DEER (*Odocoileus virginianus*)

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Abstract—Interdigital secretions were collected from eight male white-tailed deer of various ages. Analysis of volatiles was performed by gas chromatography–mass spectrometry with a modified headspace technique. Forty-six volatile compounds were found including alkanes, arenes, aldehydes, ketones, aliphatic acids, esters, pyrroles, furans, and sulfur compounds. Eleven occurred in higher concentrations ($P \leq 0.10$) in dominant ($\geq$3.5-year-old) than in subordinate ($\geq$1.5-year-old) animals. Dominant males typically have higher serum testosterone levels, and fatty acids and esters fluctuate with sebum production, which is under hormonal control. Therefore, these compounds may reflect testosterone levels and act as chemical signals indicating the presence of a dominant male. Interdigital volatiles also may act as generalized scent trail markers.

Key Words—Interdigital gland, *Odocoileus virginianus*, pheromone, scent communication, semiochemical, volatiles, white-tailed deer.

INTRODUCTION

White-tailed deer (*Odocoileus virginianus*) have been the subject of various studies involving scent communication. Deer rely on olfaction and the deposition of various semiochemicals to facilitate communication among conspecifics. These compounds can be carried on the deer or deposited in the environment

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in the form of olfactory signposts (Müller-Schwarze, 1972; Moore and Marchinton, 1974; Miller et al., 1987; Marchinton et al., 1990).

Originally described by Quay (1959), the interdigital glands are pockets between the primary digits of both fore- and hind feet. The associated skin is characterized by enlarged sebaceous glands with few apocrine glands. Enlarged erector pili muscles may act to force the glandular material out of the ducts and hair follicles. Quay (1959) reported that the sebaceous glands may enlarge with age, and the exudate may become more pigmented. Similarly, interdigital secretions from Rocky Mountain mule deer (*O. hemionus hemionus*) also become more pigmented with age (Quay and Müller-Schwarze, 1971). Pigmentation change suggests a change in chemical composition.

In reindeer (*Rangifer tarandus tarandus*), Brundin et al. (1978) found that the interdigital secretions contained several short-chain aliphatic acids, and that isovaleric and isobutyric acids elicited a behavioral response. Brundin and Andersson (1979) further found that the interdigital ketones 6-methyl-2-heptanone, 7-methyl-3-octanone, and 7-methyl-1-octen-3-one varied annually with cyclic changes in behavior and physiology.

Atkeson (1983) performed crude extractions on interdigital gland secretions of white-tailed deer and found that they contained butyric and isovaleric acids. He also suspected that acetic, propionic, and isobutyric acids were present, but these identifications were tentative. Because white-tailed deer have been observed tracking other deer and even back-tracking themselves when apparently lost (Nichol, 1938), these glands likely are responsible for leaving an olfactory trail. Few previous efforts have specifically examined the volatiles in white-tailed deer interdigital gland secretions, and those efforts were limited by the accuracy or availability of adequate analytical equipment. Therefore, we investigated the composition of the volatile compounds present in the interdigital glands and evaluated differences in the concentrations of these compounds between dominant and subordinate males.

METHODS AND MATERIALS

Secretions were collected from the interdigital glands of eight male white-tailed deer (1.5–11.5 years old). Deer were housed at the Whitehall Deer Research Facility operated by the University of Georgia's Daniel B. Warnell School of Forest Resources. They were fed a commercial feed (Checkers Pellet, Purina Mills, Inc.) and alfalfa (*Medicago sativa*) ad libitum. This provision of a similar food to all deer tested, along with extremely limited browse, helped to eliminate diet-related differences in semiochemical production. Deer were divided into several groups and placed into 1-ha pens. Does and fawns also were placed in the pens to provide possible olfactory stimuli that would induce the establishment of dominance.

Sampling of behavioral data began in October and ended December 1993. Observation periods were conducted within 2 hr after sunrise or 2 hr before sunset. The all-occurrences method described by Lehner (1979) was used due to the adequate visibility and frequency of behaviors. Matrices were then established according to Lehner (1979) to determine the social ranking of each individual within his group. At the end of the sampling period, the hierarchies were fairly stable, with all immature deer (1.5 years old, $N = 4$) being subordinate and all mature deer (3.5+ years old, $N = 4$) being dominant (Gassett, 1995).

In December 1993, deer were immobilized with a mixture of ketamine hydrochloride and xylazine hydrochloride (Mech et al., 1985). The interdigital glands of the rear foot of each deer were swabbed with medical-grade sterile cotton swabs designed for microbial sampling. Swabs were frozen immediately in glass vials at −60°C. Anesthesia was reversed with yohimbine hydrochloride.

Volatiles were removed from the swabs with a modified headspace technique (Novotny et al., 1974; McConnell et al., 1979). Swabs were placed into a headspace sampling apparatus and subjected to a stream of purified helium gas at a flow rate of 50 ml/min for 30 min at 75°C. Volatiles were passed through a cooled condenser to reduce water mist originating from the sample. Subsequently, the volatiles were trapped onto a 4-mg Tenax GC precolumn. Upon completion of the extraction, the precolumn was placed into a modified injection port of a Hewlett Packard model 5980A GC-MS. Volatiles were desorbed from the precolumn at 200°C and cryogenically retrapped for 15 min to compress the sample at the column inlet. The column used was a fused silica capillary column (Restek RTX 225, 30 m, 250 μm ID) that was provided with a cyanopropyl phase. Column temperature was programmed from 30°C to 190°C at a rate of 2°C/min. Volatile constituents were identified through their mass spectra with electron impact ionization at 70 eV. Peak areas were evaluated by a GC-MS data station. Unused swabs were subjected to the same sampling protocol to assay for background volatiles in the swabs, carrier gas, and headspace apparatus. The few background volatiles present were low enough in concentration that the interdigital assay was unaffected.

Concentrations were normalized by use of a known concentration of internal standard (7-tridecanone). Because of nonnormally distributed data, statistical comparisons of volatile concentrations between dominant and subordinate bucks were performed using a Mann-Whitney U test. Significance was accepted at $\alpha \leq 0.10$.

RESULTS AND DISCUSSION

Headspace sampling and subsequent GC-MS analysis identified 46 volatiles from interdigital secretions. The GC-MS recording shown in Figure 1 is typical of the volatile profile obtained from the analysis. The internal standard

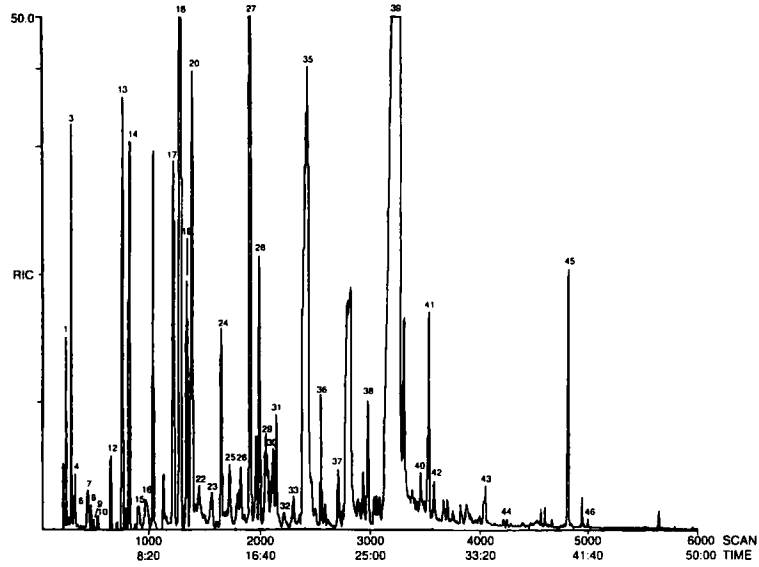

FIG. 1. Chromatogram of volatile compounds from the interdigital gland of a male white-tailed deer. The number for each identified peak corresponds with peak numbers in Table 1.

(7-tridecanone), which typically elutes between 55 and 60 min, is not shown on this figure. Representing it on the figure would have compressed the figure further and prevented the differentiation of several important compounds. It should also be noted that the compounds in Figure 1 do not coincide directly with the values in Table 1. Figure 1 is a representative chromatogram of one deer, while the values in Table 1 represent the mean for each group of deer tested. Classes of compounds identified included alkanes, arenes, aldehydes, ketones, aliphatic acids, esters, pyrroles, furans, and sulfur compounds (Table 1).

Concentrations of 11 compounds varied between dominant and subordinate animals. Because all the subordinate deer in this study were 1.5 years old and all dominant animals were ≥ 3.5 years old, we were unable to definitively conclude whether the differences in compounds were age-related or dominance-related. However, since rank typically is related to age, it is likely that both played some part in the variations.

The classes of compounds that differed included ketones, esters, arenes, alkanes, aliphatic acids, and pyrroles. Acetic acid, ethyl-2-methyl butanoate, butanone, methyl salicylate, pyrrole, pentyl butyrate, benzene, a branched

TABLE 1. MEAN PEAK AREA OF VOLATILE COMPOUNDS FROM INTERDIGITAL GLANDS OF DOMINANT (≥3.5-YEAR-OLD) AND SUBORDINATE (1.5-YEAR-OLD) MALE WHITE-TAILED DEER[a]

Compound class	Peak number	Structure	Standardized peak areas (mean ± SEM)		P
			Dominant (N = 4)	Subordinate (N = 4)	
Alkanes	1	hexane	0.051 ± 0.047	0.002 ± 0.001	0.149
	2	heptane	0.029 ± 0.027	0.002 ± 0.001	0.773
	7	octane	0.100 ± 0.093	0.004 ± 0.002	0.149
	12	nonane	0.166 ± 0.152	0.011 ± 0.002	0.663
	16	a branched decane	0.023 ± 0.019	0.001 ± 0.001	0.091
	17	decane	0.095 ± 0.084	0.003 ± 0.003	0.237
	26	undecane	0.030 ± 0.026	0.000 ± 0.000	0.091
Arenes	8	benzene	0.060 ± 0.052	0.004 ± 0.002	0.059
	14	toluene	0.434 ± 0.369	0.070 ± 0.018	1.000
	20	xylene isomer	0.753 ± 0.681	0.088 ± 0.036	0.564
	21	xylene isomer	0.149 ± 0.134	0.017 ± 0.008	1.000
	22	xylene isomer	1.551 ± 1.424	0.176 ± 0.090	0.387
	24	ethylbenzene	0.572 ± 0.529	0.056 ± 0.030	0.387
	25	3-carbon alkylbenzene	0.113 ± 0.105	0.008 ± 0.005	0.468
	27	styrene	3.389 ± 2.576	0.571 ± 0.078	0.083
	28	*n*-propylbenzene	0.180 ± 0.148	0.014 ± 0.007	0.083
	29	3-carbon alkylbenzene	0.064 ± 0.057	0.005 ± 0.003	0.564
	31	a methylstyrene	0.053 ± 0.048	0.005 ± 0.003	0.468
	34	3-carbon alkylbenzene	0.026 ± 0.013	0.003 ± 0.002	0.149
Aldehydes	3	acrolein	0.112 ± 0.092	0.017 ± 0.006	0.387
	6	butanal	0.056 ± 0.054	0.003 ± 0.001	0.564
	11	3-methylbutanal	0.127 ± 0.124	0.007 ± 0.002	0.773
	36	2-furancarboxaldehyde	0.012 ± 0.011	0.007 ± 0.006	0.767
	41	benzaldehyde	0.391 ± 0.305	0.068 ± 0.034	0.248
	42	nonanal	0.034 ± 0.025	0.015 ± 0.006	0.773

TABLE 1. CONTINUED

Compound class	Peak number	Structure	Standardized peak areas (mean ± SEM) Dominant (N = 4)	Subordinate (N = 4)	P
Ketones	4	acetone	0.235 ± 0.199	0.026 ± 0.009	0.248
	9	butanone	0.014 ± 0.011	0.001 ± 0.001	0.042
	10	butenone	0.054 ± 0.049	0.005 ± 0.003	0.564
	44	acetophenone	0.017 ± 0.014	0.003 ± 0.002	0.309
	47	7-tridecanone (internal standard)	1.000 ± 0.000	1.000 ± 0.000	1.000
Sulfur compound	15	dimethyl disulfide	0.038 ± 0.038	0.001 ± 0.001	0.767
Furans	5	2-methylfuran	0.043 ± 0.043	0.000 ± 0.000	0.317
	30	2-pentylfuran	0.043 ± 0.041	0.001 ± 0.001	0.166
Pyrroles	32	pyrrole	0.047 ± 0.020	0.002 ± 0.002	0.042
	37	2-methylpyrrole	0.071 ± 0.051	0.011 ± 0.009	0.663
Alcohols	40	2-ethyl-1-hexanol	0.069 ± 0.060	0.017 ± 0.006	0.885
Aliphatic acids	23	acetic acid	0.056 ± 0.044	0.000 ± 0.003	0.018
	35	isobutyric acid	1.653 ± 1.113	1.306 ± 0.953	0.387
	39	3-methylbutanoic acid	1.367 ± 0.371	1.491 ± 0.986	0.564
Esters	13	ethyl butyrate	0.148 ± 0.138	0.007 ± 0.002	0.564
	18	ethyl 2-methylbutanoate	0.039 ± 0.030	0.000 ± 0.000	0.021
	19	ethyl pentanoate	0.285 ± 0.229	0.039 ± 0.027	0.248
	33	phentyl butyrate	0.148 ± 0.074	0.030 ± 0.028	0.083
	38	pentyl pentanoate	0.391 ± 0.302	0.171 ± 0.164	0.663
	43	methyl benzoate	0.007 ± 0.005	0.005 ± 0.003	0.885
	45	ethyl benzoate	0.833 ± 0.402	0.502 ± 0.343	0.564
	46	methyl salicylate	0.021 ± 0.012	0.001 ± 0.001	0.018

[a] Values represent concentrations relative to the standard (7-tridecanone). Samples were collected at the University of Georgia's Whitehall Deer Research Facility in December 1993.

decane, undecane, styrene, and an unidentified arene all occurred in greater concentrations in dominant than subordinate males ($P \leq 0.10$). Many of these compounds commonly are associated with lipid secretion. Because androgenic hormones affect sebum production, and testosterone levels are generally higher in dominant males, the variation between social classes could indicate that these compounds act as dominance-specific semiochemicals.

The short-chain fatty acids we found may have resulted from microbial hydrolysis of triglyceride ester linkages. Microbes can break triglyceride ester linkages to release fatty acids as by-products. However, the esters we found in the interdigital secretions (Table 1) are similar to those found in the seba of most mammalian species and are not hydrolyzed easily by microbial action (Albone, 1984). Thus, the esters and fatty acids probably differ in their origins, but both groups likely are present as the result of secretions of the sebaceous glands.

The presence of androgens markedly increases sebaceous gland size and sebum production (Albone, 1984). Because androgen levels are higher in dominant male white-tailed deer during the breeding season (Miller et al., 1987), deer with higher androgen levels would be expected to produce larger quantities of interdigital fatty acids and esters. In a behavioral study of reindeer interdigital gland secretions, Müller-Schwarze et al. (1978) found that animals were more attracted to secretions from animals of the same sex, but the male scent elicited stronger behavioral responses. Social or age-related variations in the secretions may give the dominant animals a stronger scent and could act as a signal to females and subordinate males that a dominant male is in the area.

The remaining background volatiles secreted from the interdigital glands are produced at a constant rate and could possibly act to establish trails between bedding, feeding, and shelter areas. These compounds are of a volatility that would allow them to remain in the environment for several hours (or longer in a lipid matrix).

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FIELD ATTRACTION OF *Biprorulus bibax* BREDDIN (HEMIPTERA: PENTATOMIDAE) TO SYNTHETIC AGGREGATION PHEROMONE AND (*E*)-2-HEXENAL, A PENTATOMID DEFENSE CHEMICAL

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Abstract—A synthetic blend of the aggregation pheromone [(3*R*,4*S*,1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol, linalool, farnesol, and nerolidol] of the spined citrus bug, *Biprorulus bibax*, and the pentatomid defense chemical, (*E*)-2-hexenal, both attracted adult *B. bibax* to individual trees in citrus orchards. Lemon trees containing single glass vials with aggregation pheromone or (*E*)-2-hexenal were colonized by significantly greater numbers of reproductive *B. bibax* than unbaited trees. There was no significant difference between the treatments and bug recruitment was not improved by using both treatments. *B. bibax* did not enter cylinder/funnel traps baited with aggregation pheromone but colonized trees containing the traps. Orange or lemon trees containing aggregation pheromone on orchard perimeters recruited significantly larger populations of emigrating, nonreproductive *B. bibax* during fall than untreated trees. Nonreproductive bugs were not attracted to trees containing (*E*)-2-hexenal. The potential for using these semiochemicals as management tools for *B. bibax* is discussed.

Key Words—Aggregation pheromone, (*E*)-2-hexenal, *B. bibax*, citrus, Pentatomidae.

INTRODUCTION

The spined citrus bug, *Biprorulus bibax* Breddin (Hemiptera: Pentatomidae), is an important pest of citrus in inland southeastern Australia causing internal

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staining/drying of fruit and fruit drop (Hely et al., 1982; James, 1994). Recent research on the biology and ecology of this native insect (e.g., James, 1990a–c, 1991; James et al., 1990) led to development and adoption of an integrated management strategy based on monitoring, natural enemy conservation, and judicious use of the insecticide, endosulfan, at a rate harmless to natural enemy survival (James, 1993, 1994). However, endosulfan is likely to be removed from use as a horticultural pesticide in Australia in the near future and an alternative control tactic is required.

Studies on the pheromone biology of *B. bibax* commenced after James and Warren (1989) reported the presence of sexual dimorphism in the dorsal abdominal glands (DAGs) of adult bugs. The enlarged DAGs of male *B. bibax* contain the novel hemiacetal, (3*R*,4*S*,1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol, linalool, two isomers of farnesol, and nerolidol (Oliver et al., 1992; Mori et al., 1992, 1993). The small DAGs of female *B. bibax* contain only linalool. James et al. (1994), in a shade house bioassay permitting flight, demonstrated significant attraction of postdiapause, prereproductive, and reproductive female *B. bibax* to heptane extracts of male DAGs and a synthetic blend of the male DAG secretion. Mating leks have been observed in *B. bibax* (James, 1989; James and Warren, 1989), indicating both sexes respond to the pheromone, similar to the situation reported for the North American predaceous pentatomid, *Podisus maculiventris* (Say) (Aldrich et al., 1984). Shade house bioassays have also demonstrated attraction of *B. bibax* to (*E*)-2-hexenal (James, unpublished observations), a major component of the defensive secretion of *B. bibax* and many other pentatomid species (MacLeod et al., 1975; Aldrich, 1988). A synthetic pheromone-trapping system would substantially improve the current monitoring system for *B. bibax* (tree searches) and might also have potential as the basis of a control tactic (e.g., mass trapping) to replace the use of endosulfan.

This study provides information on the field response of *B. bibax* to synthetic aggregation pheromone and (*E*)-2-hexenal.

METHODS AND MATERIALS

Five experiments to evaluate the field response of adult *B. bibax* to a synthetic blend of aggregation pheromone and (*E*)-2-hexenal were conducted in four mature commercial citrus orchards in the Yanco-Narrandera district of southern New South Wales during February–May (late summer–late fall) 1995. The aggregation pheromone was formulated to approximate the composition of male DAG extracts and extracts of volatiles from live males (hemiacetal 46%, linalool 40%, farnesol 13%, nerolidol 1%) (James et al., 1994). The racemate of (3*R*,4*S*,1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol was synthesized in Japan (Mori et al., 1992) and shipped to Australia. The other components of the

pheromone were obtained from Sigma-Aldrich Pty. Ltd. (Castle Hill, New South Wales). All components (hemiacetal 46 mg, linalool 40 mg, farnesol 13 mg, nerolidol 1 mg) were blended in 3 ml of heptane in 5-ml glass vials and stored in a freezer until used (within 48 hr of preparation). Uncapped vials were taped to interior (shaded) tree branches or placed in traps and replaced weekly. Approximately 10 mg of pheromone per vial was released daily.

(*E*)-2-Hexenal was obtained from Sigma-Aldrich and used in uncapped glass vials (300 mg/vial) taped to tree branches. Release rates were approximately 100–200 mg/day (according to weather conditions). Vials were replaced daily due to the rapid oxidation of (*E*)-2-hexenal on exposure to air/light.

Experiment 1. This was conducted in a 1.5-ha lemon [*Citrus limon* (L.) Burm. f.] (var. Eureka) orchard at Cudgel near Narrandera, New South Wales, from February 14 to March 3 to investigate the effect of (*E*)-2-hexenal on reproductive adult *B. bibax*. Twenty trees, evenly distributed throughout the block, were selected and assigned as treatment ($N = 10$) or nontreatment ($N = 10$). All of the treatment trees were in the bottom half of the block and the nontreatment trees in the top half. A minimum distance of 10 m separated each tree marked by colored tape. On February 14, a pretreatment count of adult *B. bibax* on all marked trees was conducted with approximately 2–3 min spent scanning fruit and foliage on the outside of the tree canopy, without disturbing the bugs. Subsequent counts in all experiments were conducted by the same observer in the same way. Treatment trees were baited with a single vial of (*E*)-2-hexenal. Posttreatment counts were conducted on 13 of the following 20 days.

Experiment 2. Conducted during March 6–17, in the same orchard as experiment 1, this experiment looked at the individual effects and combined effect of (*E*)-2-hexenal and aggregation pheromone on recruitment of reproductive adult *B. bibax* to individual trees. The same trees were used as in experiment 1 with five control trees in one top quarter of the block assigned to an aggregation pheromone + (*E*)-2-hexenal treatment (one vial of each per tree), while the other five remained as untreated controls. In the bottom half of the block, five trees remained as (*E*)-2-hexenal-treated while the other five were assigned to aggregation pheromone only treatment. Pretreatment counts were conducted on March 6, prior to commencement of baiting. Posttreatment counts were conducted on nine of the 11 days following initiation of treatments.

Experiment 3. This experiment was conducted during March 17–23, in the first row of oranges [*Citrus sinensis* (L.) Osbeck] (var. Valencia), adjacent to the lemon orchard used in the previous experiments, to determine whether reproductive adult *B. bibax* would enter cylinder/funnel traps baited with aggregation pheromone. Cylinder/funnel traps were constructed from Perspex cylinders (27 × 15 cm) with two holes (4 cm diam.) on opposite sides. Each hole was covered by an inwardly projecting screen funnel. A single trap was used in each tree

and hung from an interior (shaded) branch approximately 1.5 m above ground level. Three traps were baited with aggregation pheromone (vial taped to interior surface of the trap) and placed in trees adjacent to the section of the lemon orchard used as an untreated area in earlier experiments. Three unbaited traps were placed in trees adjacent to the aggregation pheromone-treated area of experiment 2. No bugs were found in any of the six trees prior to baiting. Posttreatment counts of adult *B. bibax* in traps or on the trees in which the traps were hung were conducted on the final three days of the six-day period following treatment.

Experiment 4. The experiment was conducted during April 4–May 29, in a different commercial lemon orchard (2 ha) near Narrandera, to determine the effect of aggregation pheromone and (*E*)-2-hexenal on recruitment of nonreproductive adult *B. bibax* to individual trees for overwintering. In late March–early April adult *B. bibax* enter reproductive dormancy (James, 1991) and usually leave lemon orchards to overwinter in large aggregations (50–500 individuals per tree) in adjacent nonlemon citrus, usually oranges (James, 1989, 1990a,b; James et al., 1990). This experiment commenced prior to development of overwintering aggregations and ran during the period when most colonization of overwintering sites occurred. A large population of reproductive *B. bibax* (40–60 adults found in an hour's search on March 24) occurred in this lemon orchard prior to commencement of the experiment. The orchard was bordered on three sides by oranges, the first row of which harbored large numbers of overwintering *B. bibax* in previous seasons (James, unpublished observations). Nine orange trees (three on each side of the lemon orchard) were selected, marked, and assigned (three replicates) as aggregation pheromone, (*E*)-2-hexenal, or no treatment. Both treatments and the control were represented on the three sides of the lemon orchard and a minimum distance of 20 m separated treatment and control trees. No bugs were present in any of the nine trees prior to treatments, which began on April 4. Posttreatment counts were conducted on 26 of the 55 days following initiation of treatments.

Experiment 5. These tests were conducted in parallel with experiment 4 in a 0.25-ha lemon orchard and a nearby (20 m) orange orchard (0.5 ha) at the Yanco Agricultural Institute, again to determine the effect of aggregation pheromone and (*E*)-2-hexenal on recruitment of nonreproductive *B. bibax* to individual trees for overwintering. *B. bibax* was not present in either of these orchards, so 1000 nonreproductive adult *B. bibax* collected from district orchards were released into the center of the lemon block on April 3. Treatment of three trees in the outside row on two sides of the block with either aggregation pheromone, (*E*)-2-hexenal, or no treatment commenced on April 4. The six trees were separated by at least 7 m and contained no bugs prior to treatment. Six trees in the two outside rows of the nearby orange block were treated in the same way. Posttreatment counts were conducted in both blocks on 26 of the 55

days following initiation of treatments. Although most *B. bibax* overwinter on nonlemon citrus, individuals can overwinter successfully on lemon (James et al., 1990).

All data were transformed to the log $(X + 1)$ scale and subjected to analysis of variance and least significant difference procedures.

RESULTS

Experiment 1. Numbers of adult reproductive *B. bibax* on trees treated with (*E*)-2-hexenal were significantly greater (mean: 2.5 ± 0.2 bugs/tree/day) than on untreated trees (1.3 ± 0.1) ($P < 0.05$). Significantly greater numbers of bugs occurred on the treated trees on all occasions except pretreatment, the day following first treatment, and on February 20 and 22 ($P < 0.05$) (Figure 1).

Experiment 2. Numbers of adult reproductive *B. bibax* on trees treated with (*E*)-2-hexenal, aggregation pheromone, or (*E*)-2-hexenal + aggregation pheromone were significantly greater (means: 6.5 ± 0.6, 8.2 ± 1.2, and 7.7 ± 1.6 bugs/tree/day, respectively) than on untreated trees (1.9 ± 0.4) ($P < 0.05$). There was no significant difference between the three experimental treatments ($P > 0.05$). Significantly greater numbers of bugs occurred on the trees treated with aggregation pheromone and aggregation pheromone + (*E*)-2-hexenal than on untreated trees on all occasions except pretreatment and the day following

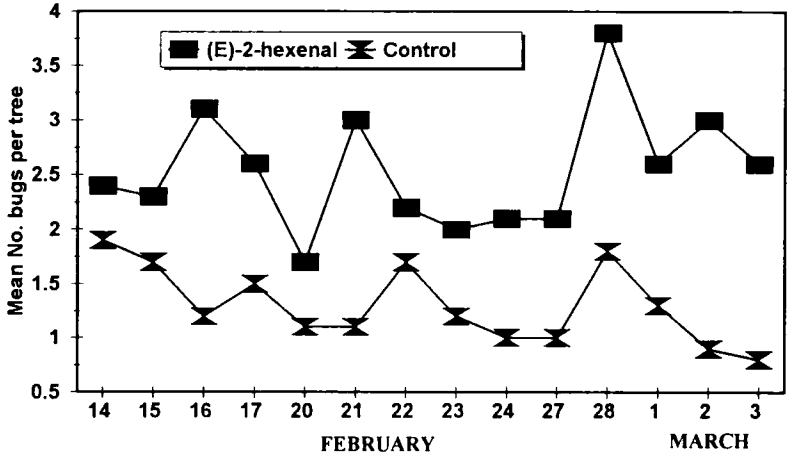

FIG. 1. Abundance of reproductive adult *B. bibax* on lemon trees treated with (*E*)-2-hexenal compared to untreated trees (control). Treatment commenced on February 14 after pretreatment counts. Significantly greater numbers of bugs occurred on treated trees on all posttreatment dates except February 15, 20, and 22 ($P < 0.05$).

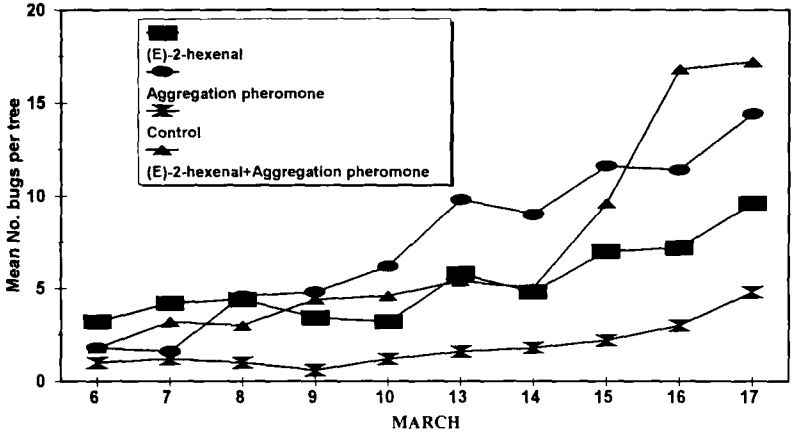

FIG. 2. Abundance of reproductive adult *B. bibax* on lemon trees treated with (*E*)-2-hexenal, aggregation pheromone, and (*E*)-2-hexenal + aggregation pheromone compared to untreated trees (control). Treatments commenced on March 6, after pretreatment counts. Significantly greater numbers of bugs occurred on trees treated with aggregation pheromone or aggregation pheromone + (*E*)-2-hexenal than on untreated trees on all posttreatment dates ($P < 0.05$). Significantly greater numbers of bugs occurred on (*E*)-2-hexenal treated trees on all posttreatment dates except March 10, 14, and 17 ($P < 0.05$).

first treatment ($P < 0.05$) (Figure 2). Significantly greater numbers of bugs were found on (*E*)-2-hexenal-treated trees than on untreated trees on all but three occasions (March 10, 14, 17) ($P < 0.05$).

Experiment 3. No bugs were trapped but significantly greater numbers occurred on aggregation pheromone-treated trees (27 ± 5/bugs/tree) than on untreated trees (4.3 ± 0.7) ($P < 0.05$). From no bugs pretreatment, treated trees recruited 22–54 bugs per tree within five days, while untreated trees gained 1–6 per tree (Figure 3).

Experiment 4. Aggregation pheromone-treated trees recruited significantly greater numbers of nonreproductive *B. bibax* (69 ± 5.4 bugs/tree/day) than either (*E*)-2-hexenal-treated (1.2 ± 0.2) or untreated trees (3.9 ± 0.6) ($P < 0.05$). There was no significant difference between (*E*)-2-hexenal-treated and untreated trees ($P > 0.05$). Up to 168 bugs were counted on individual aggregation pheromone-treated trees during May while (*E*)-2-hexenal-treated and untreated trees harbored a maximum of 10 and 21 bugs per tree, respectively. Significant recruitment to the pheromone-treated trees commenced around mid-April and continued for about four weeks (Figure 4).

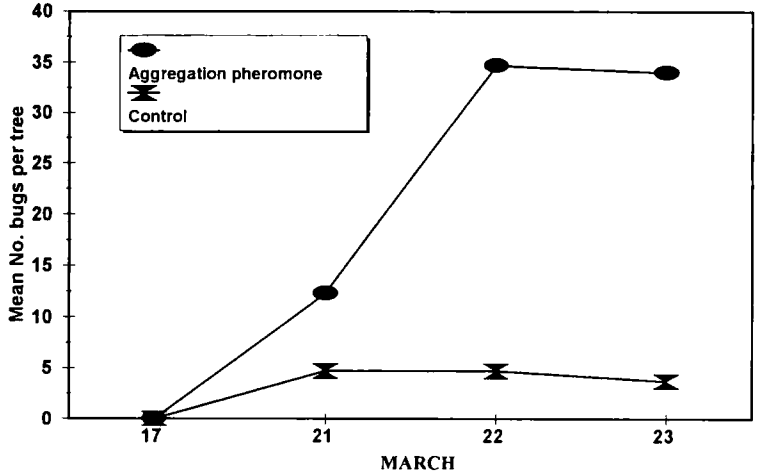

FIG. 3. Abundance of reproductive adult *B. bibax* on orange trees containing cylinder/funnel traps baited on March 17 with aggregation pheromone and on trees without traps (control). Significantly greater numbers of bugs occurred on trees with traps on all post-treatment dates ($P < 0.05$).

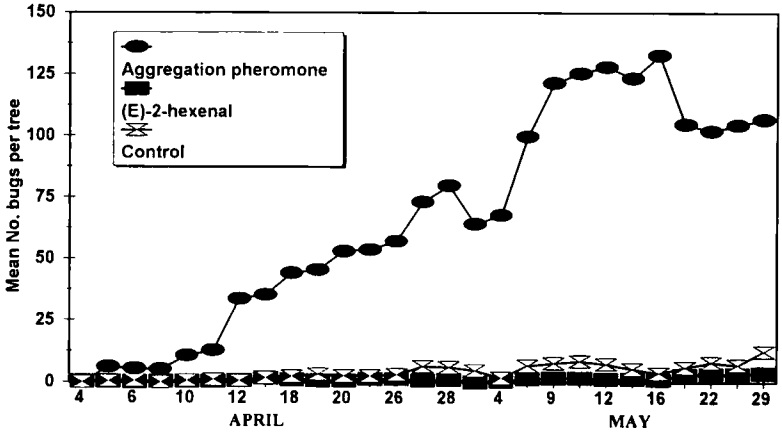

FIG. 4. The effect of aggregation pheromone and (*E*)-2-hexenal on fall colonization of orange trees on the perimeter of a lemon orchard by non-reproductive adult *B. bibax* seeking overwintering sites.

Experiment 5. Aggregation pheromone-treated trees recruited significantly greater numbers of nonreproductive *B. bibax* in the lemon block (5.1 ± 0.6 bugs/tree/day) and orange block (1.5 ± 0.3) than either (*E*)-2-hexenal-treated trees (lemon: 0.04 ± 0.03; orange: 0.06 ± 0.03) or untreated trees (lemon: 0.02 ± 0.02; orange: nil) ($P < 0.05$). No significant difference occurred between (*E*)-2-hexenal-treated and untreated trees in either block ($P > 0.05$). Recruitment to pheromone-treated trees, particularly in the lemon block, followed a similar pattern to that observed in experiment 4 with maximum numbers of bugs in mid-May (Figure 5).

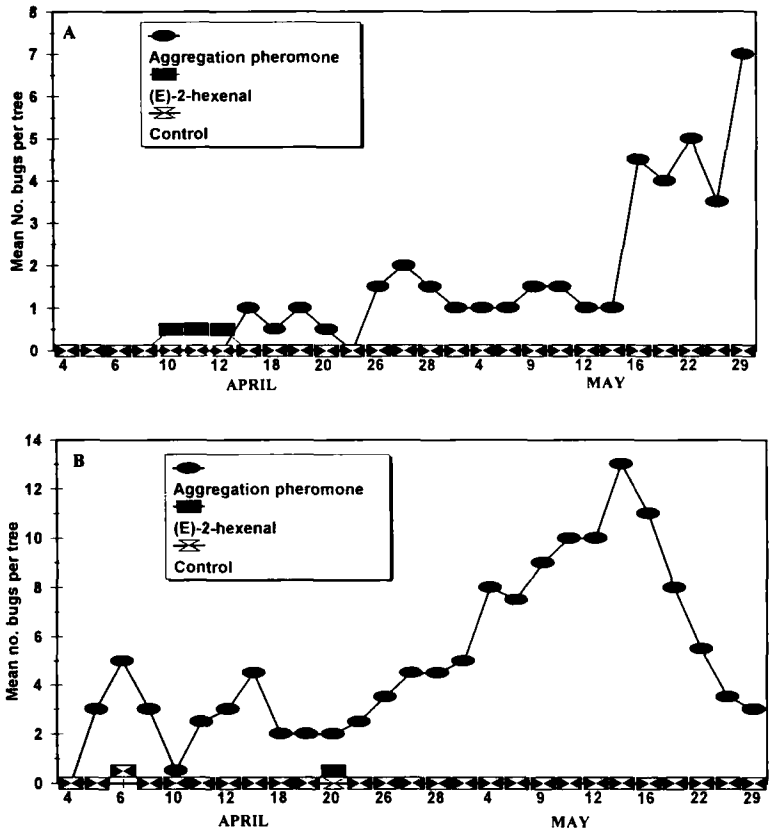

FIG. 5. The effect of aggregation pheromone and (*E*)-2-hexenal on fall colonization of orange (A) and lemon (B) trees by nonreproductive *B. bibax* seeking overwintering sites.

DISCUSSION

This study demonstrates synthetic aggregation pheromone to be a potent attractant for reproductive and nonreproductive *B. bibax* in the field. In addition, it provides field evidence for (*E*)-2-hexenal, a major component of stink bug defense chemistry, as an attractant for *B. bibax* in its own right.

Synthetic aggregation pheromone released from single glass vials at a rate of approximately 10 mg/day significantly increased the colonization of citrus trees by reproductive and nonreproductive *B. bibax*. Although no accurate counts were made, males and females occurred on treated trees in similar numbers. In earlier studies (James et al., 1994), reproductive and prereproductive female *B. bibax* were significantly attracted to synthetic aggregation pheromone in shade house experiments, although nonreproductive females collected from fall populations were not. The impressive recruitment of *B. bibax* to pheromone-treated trees on the periphery of colonized blocks during fall (particularly in experiment 4) was a convincing demonstration of the potency of aggregation pheromone to diapausing individuals, and the disparity between shade house and field results is difficult to explain. The pheromone-producing DAGs of diapausing male *B. bibax* are smaller than those of reproductive males but larger than those of teneral males (James and Warren, 1989), indicating a role for aggregation pheromone in the formation and maintenance of overwintering colonies (James, 1990a). The colonies of *B. bibax* in pheromone-treated trees often formed dense clusters like naturally occurring overwintering aggregations (James, 1990a), although populations were undoubtedly underestimated because bugs within canopies were not counted.

Although *B. bibax* were readily attracted to trees containing pheromone, they did not enter pheromone-baited cylinder/funnel traps. Pheromone traps of a similar design have successfully trapped smaller pentatomids (e.g., Aldrich et al., 1984), and it is possible that the less agile flight of the relatively large and heavy *B. bibax* (James et al., 1990) prevented direct location of trap entry holes. The relatively high release rate of pheromone (and therefore high content in air around the tree) may have inhibited further searching by bugs for a source point after alighting on the tree. This was supported by occasional observations on trees adjacent to pheromone-treated trees that invariably indicated bug populations almost as large as on the pheromone-treated tree. The possibility that a lower rate of pheromone release might overcome this problem should be investigated.

Trees treated with (*E*)-2-hexenal developed populations of reproductive *B. bibax* two to three times larger than those of untreated trees. In experiment 2, where the effect of aggregation pheromone and (*E*)-2-hexenal were compared, there was no difference in bug response to these treatments. However, unlike

aggregation pheromone, there was no response to (*E*)-2-hexenal by nonreproductive *B. bibax*.

The most notable function of (*E*)-2-hexenal in pentatomid and, indeed, heteropteran biology, is as a major component of defensive secretions active against invertebrate predators such as ants (Aldrich, 1988). It also acts as an alarm pheromone, alerting conspecifics to danger and stimulating dispersal (Ishiwatari, 1974, 1976; Lockwood and Story, 1987). However, Ishiwatari (1976) showed (*E*)-2-hexenal was bifunctional in its effect on first-instar nymphs of *Eurydema rugosa* Motschulsky; large quantities (>0.05% in water) caused dispersal while smaller quantities enhanced aggregation of the nymphs. Lockwood and Story (1985) demonstrated a similar bifunctionality for *n*-tridecane, another important component of pentatomid defensive secretions, in first instars of *Nezara viridula* (L.). Interestingly, (*E*)-2-hexenal is an important component of the aggregation pheromones of some predatory pentatomids in the genus *Podisus* (Aldrich, 1995). However, the current study is the first to demonstrate field attraction of an adult pentatomid to (*E*)-2-hexenal alone. Point sources dispensing 100–200 mg of (*E*)-2-hexenal per day produced an atmospheric concentration that clearly encouraged tree colonization by reproductive adult *B. bibax*. In contrast, close-range exposure of adult *B. bibax* to (*E*)-2-hexenal in the laboratory induces dispersal behavior (James, unpublished observations), as it does with many other pentatomids (e.g., Lockwood and Story, 1987). These observations support Ishiwatari's (1976) conclusions on dose-determined bifunctionality of (*E*)-2-hexenal for first-instar nymphs of *E. rugosa*. Aldrich (1995) suggested that the major hemiacetal of the aggregation pheromone of *B. bibax* may be formed by enzymatic dimerization of 2-hexenal, "extending an ancient biosynthetic pathway." If this is the case, then (*E*)-2-hexenal may be the ancestral aggregation pheromone for *B. bibax*, which evolved into the distinctive hemiacetal-dominated pheromone that presumably has major functional advantages (e.g., lower volatility, slower degradation, increased efficacy over long distances). (*E*)-2-Hexenal is an ancient compound; small amounts occur as a volatile in most green leaves (leaf aldehyde). *B. bibax* also may use (*E*)-2-hexenal as a cue to locate host plants and/or to synergize the response to pheromone.

Synthetic aggregation pheromone and (*E*)-2-hexenal clearly deserve further study as potential tools for use in orchard management of *B. bibax*. The aggregation pheromone is an effective attractant for reproductive and nonreproductive adults, and further research should concentrate on developing pheromone-based management strategies. For example, the pheromone could be used to divert/congregate bugs in selected areas for targeted chemical control. Pheromone-based trapping of *B. bibax* either for monitoring or control should be investigated further. Traps that require minimal navigational skills for entry by *B. bibax* (e.g., upward-facing funnel or water-pan traps) and lower pheromone release

rates should be tested. Despite improvements to synthesis of the hemiacetal component of the aggregation pheromone (Mori et al., 1993), it is still relatively difficult and expensive to produce. While (*E*)-2-hexenal requires a lot more field evaluation to determine its merits and potential use relative to that of the aggregation pheromone (its noneffect on nonreproductive bugs is a drawback), it does have the advantage of being readily available. The problems of rapid degradation and high volatility should be resolvable by using antioxidants and slow-release devices.

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AVOIDANCE RESPONSE OF A TERRESTRIAL SALAMANDER (*Ambystoma macrodactylum*) TO CHEMICAL ALARM CUES

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Abstract—Organisms from a wide variety of taxonomic groups possess chemical alarm cues that are important in mediating predator avoidance. However, little is known about the presence of such alarm cues in most amphibians, and in particular terrestrial salamanders. In this study we tested whether adult long-toed salamanders (*Ambystoma macrodactylum*) showed an avoidance response to stimuli from injured conspecifics. Avoidance of stimuli from injured conspecifics could represent avoidance of a chemical alarm cue or, alternatively, avoidance of a territorial pheromone or conspecific predator odor. Consequently, we also tested whether salamanders avoided stimuli from noninjured conspecifics. Salamanders avoided stimuli from injured but not from noninjured conspecifics. Therefore, we concluded that the response to injured conspecifics represents avoidance of a chemical alarm cue and not avoidance of a territorial pheromone or predator cue. This is the first clear demonstration of chemical alarm signaling by a terrestrial amphibian and the first report of chemical alarm signaling in an ambystomatid salamander. By avoiding an area containing stimuli from injured conspecifics, long-toed salamanders may lower their risk of predation by avoiding areas where predators are foraging.

Key Words—Alarm pheromone, chemical signaling, predator avoidance, long-toed salamanders, *Ambystoma macrodactylum*.

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INTRODUCTION

A wide variety of taxa have been shown to detect and respond to chemical alarm signals (alarm pheromones) released by conspecifics. Such signals can be released by the sender either before or after a predator captures the prey (e.g., Smith, 1992; Wisenden et al., 1995a,b). Signals that are released upon mechanical damage to the sender (known as damage-release alarm signals) should act as reliable indicators of predation risk in that they indicate that a conspecific in the immediate vicinity was recently attacked or captured by a predator. By avoiding areas where predators have recently captured a conspecific, prey should be able to lower their probability of being captured.

Damage-release chemical alarm signals have been documented in various taxa (insects: Sih, 1986; Chivers et al., 1996; gastropods: Snyder, 1967; Appleton and Palmer, 1988; echinoderms: Parker and Schulman, 1986; Lawrence, 1991; sea anemones: Howe and Sheikh, 1975; crayfish; Hazlett, 1994; fishes: Smith, 1992; amphibians: see below). Except for fish, there is relatively little information on the distribution of alarm pheromones among the vertebrates. For example, few studies have been conducted on amphibian alarm pheromones. Eible-Eibesfeldt (1949), Hrbacek (1950), and Kulzer (1954) described an alarm response to stimuli from injured conspecifics in common toad (*Bufo bufo*) tadpoles. In laboratory experiments, tadpoles exposed to conspecific stimuli typically swam to the bottom or to the side of the tank opposite from where the pheromone was introduced. In field experiments, tadpoles avoided alarm pheromones by swimming into deeper water. Pfeiffer (1966) tested nine species of larval anurans in five different families. He found responses in two species of bufonid tadpoles (*Bufo bufo* and *B. calamita*), and speculated that alarm pheromones may be restricted to the family Bufonidae. Alarm pheromones are also known from another bufonid, the western toad (*Bufo boreas*) (Hews and Blaustein, 1985; Hews, 1988). Recent studies have shown that alarm pheromones may also be found in larval ranid frogs, including Cascades frog tadpoles (*Rana cascadae*) (Hews and Blaustein, 1985, but see Hokit and Blaustein, 1996) and red-legged frog tadpoles (*Rana aurora*) (Wilson and Lefcort, 1993).

Only two studies have investigated the possibility of chemical alarm signals in salamanders. Lutterschmidt et al. (1994) showed that adult plethodontids, *Desmognathus ochrophaeus*, avoid skin extracts from conspecifics and congeners, *D. brinleyorum*. This may represent an avoidance response to chemical alarm cues. However, this response may also represent avoidance of territorial pheromones or predator odors. Other plethodontid salamanders have been shown to avoid territorial pheromones and odors of predatory plethodontids (e.g., Jaeger, 1986; Roudebush and Taylor, 1987). In another experiment that tested for the presence of alarm signals in salamanders, Marvin and Hutchison (1995)

demonstrated that two species of adult semiaquatic newts (fire-bellied newts, *Cynops pyrrhogaster* and red-spotted newts, *Notophthalmus viridescens*) avoided the area of a test chamber containing moistened paper towel that was saturated with skin extracts from conspecifics. Since adult newts are not territorial and are not preyed upon by other newts or salamanders, Marvin and Hutchison (1995) concluded that the avoidance response represented avoidance of a chemical alarm cue.

In this study we investigated the avoidance response of the terrestrial form of the long-toed salamander (*Ambystoma macrodactylum*) to stimuli from injured conspecifics. Although adult long-toed salamanders are not known to be territorial or to prey on conspecifics, we also tested the salamanders for an avoidance response to chemical stimuli from noninjured conspecifics. An avoidance response to stimuli from noninjured conspecifics may indicate avoidance of territorial pheromones or conspecific predator odors and would complicate our interpretation of an avoidance response to stimuli from injured conspecifics. However, if adult long-toed salamanders are shown to avoid chemical cues from injured conspecifics but not from noninjured conspecifics, our study would be the first to clearly show chemical alarm signaling by a terrestrial amphibian.

METHODS AND MATERIALS

Collection and Maintenance. In November 1995, we collected adult long-toed salamanders on or beside a 200-m section of Oakville road, located approximately 8 km east of Corvallis, Benton County, Oregon. Each salamander was maintained individually in a Petri dish (150 mm diameter × 25 mm deep) that contained a moistened paper towel. The salamanders were maintained on a 14L:10D photoperiod at approximately 16°C and were periodically fed with crickets. The salamanders were not fed for three days prior to testing.

Experimental Protocol. We tested the avoidance response of individual salamanders to three different stimuli: (1) chemical stimuli from injured conspecifics, (2) chemical stimuli from noninjured conspecifics, and (3) a combination of chemical stimuli from both injured and noninjured conspecifics. Testing the response of salamanders to a combination of stimuli from injured + noninjured conspecifics was done in order to allow us to speculate on the strength of an avoidance response to either injured or noninjured stimuli. To prepare the injured conspecific stimulus we killed six animals by decapitation and removed their viscera, spinal column, and legs. The remaining tissue (approximately 12.0 g, which contained mostly skin, but also some muscle tissue) was ground with a mortar and pestle, combined with 200 ml of dechlorinated tap water and filtered through a fine mesh net. We used 5 ml of injured conspecific stimulus per trial.

This solution was used within 15 min of preparation. For a source of chemical stimuli from noninjured conspecifics, we used moistened paper towels that had housed a single conspecific for 48 hr.

For each trial we lined half of a rectangular plastic container (32 × 18 × 8 cm) with a paper towel that was moistened with dechlorinated tap water (control side). The treatment side of the containers was lined with paper towel that contained stimuli from injured conspecifics, stimuli from noninjured conspecifics, or a combination of both. The two paper towel substrates in each container were separated by approximately 2 cm to reduce diffusion of chemicals between the sides. After the appropriate stimuli were added to each side of the test containers, we used a spray bottle containing dechlorinated tap water to saturate the paper towels. This ensured that any observed avoidance of the control or stimulus sides could not be attributed to differences in moisture level between the sides.

At the start of each trial, we introduced a single test salamander into the center of the container. Every 3 min for 2 hr we recorded whether the test salamander was located on the control or treatment side of the container. If the salamanders were positioned across the middle of the containers, we used the position of the salamanders' snout to assign location. To control for the possibility of a bias in the salamanders' orientation in the room, we rotated the containers 180° every half hour during the experiment.

Twenty different salamanders were tested in each of the three treatments for a total of 60 tests in this experiment. Individual salamanders were never used in more than one test. We size-matched the salamanders for tests in the three treatments. The snout–vent length of salamanders was 45.0 ± 7.9 mm (mean ± SD). For each trial we summed the number of times each salamander was located on the treatment side of the container out of a possible 40 observations (one observation every 3 min for 120 min = 40 observations). For each of the three treatments, we used a binomial test (Siegel and Castellan, 1988) to compare the number of trials in which the salamanders spent less than 50% of their time on the stimulus side.

RESULTS

In trials using stimuli from noninjured conspecifics, there was no difference in the number of trials in which test salamanders chose the control side over the stimulus side of the testing chamber (Table 1, Figure 1). However, in both the injured conspecific and injured + noninjured conspecific treatments, the test salamanders showed a significant avoidance of the treatment side of the containers (Table 1, Figure 1).

TABLE 1. NUMBER OF TRIALS IN WHICH TEST SALAMANDERS CHOSE STIMULUS AND CONTROL SIDES OF EXPERIMENTAL CONTAINERS[a]

Treatment	Trials (N) where test animals spent majority of time		P
	Stimulus side	Control side	
Noninjured	12	8	0.504
Noninjured + injured	5	15	0.042
Injured	3	17	0.002

[a]See text for details, P values are based on two-tailed binomial tests.

DISCUSSION

The results of our study show that adult long-toed salamanders avoid chemical stimuli from injured conspecifics. Given that salamanders did not avoid stimuli from noninjured conspecifics, the avoidance response to injured conspecific stimuli cannot be interpreted as an avoidance response to territorial pheromones or predator odors. As a result, we conclude that the avoidance response of the salamanders to injured conspecifics represents avoidance of a chemical alarm cue. This is the first clear demonstration of chemical alarm signaling in a terrestrial amphibian and the first report of chemical alarm signaling in an ambystomatid salamander.

We found that salamanders exposed to stimuli from injured conspecifics spent more time on the control (dechlorinated water) side of the testing chamber than salamanders exposed to stimuli from injured + noninjured conspecifics. The intermediate response of salamanders to the combined stimuli from injured + noninjured conspecifics, in relation to either the noninjured or injured treatments alone, indicates that the salamanders may have a conflicting response to the combined stimuli. In the combined treatment, the presence of noninjured stimuli appeared to have weakened the intensity of the avoidance response to the injured conspecific stimuli. Nevertheless, salamanders still exhibited a significant avoidance of alarm cues in the combined stimulus treatment.

We do not know the identity of the chemical(s) that act as the alarm cue in long-toed salamanders. Nevertheless, the cue is likely contained in the skin (and/or possibly muscle tissue) as these were the tissues we used to prepare the injured conspecific stimulus. Kulzer (1954) suggested that bufotoxin, a predator deterrent contained in the skin of bufonid tadpoles, may serve as the alarm cue for bufonid tadpoles. In his study, bufotoxin (bufodienolides) and γ-bufotoxin

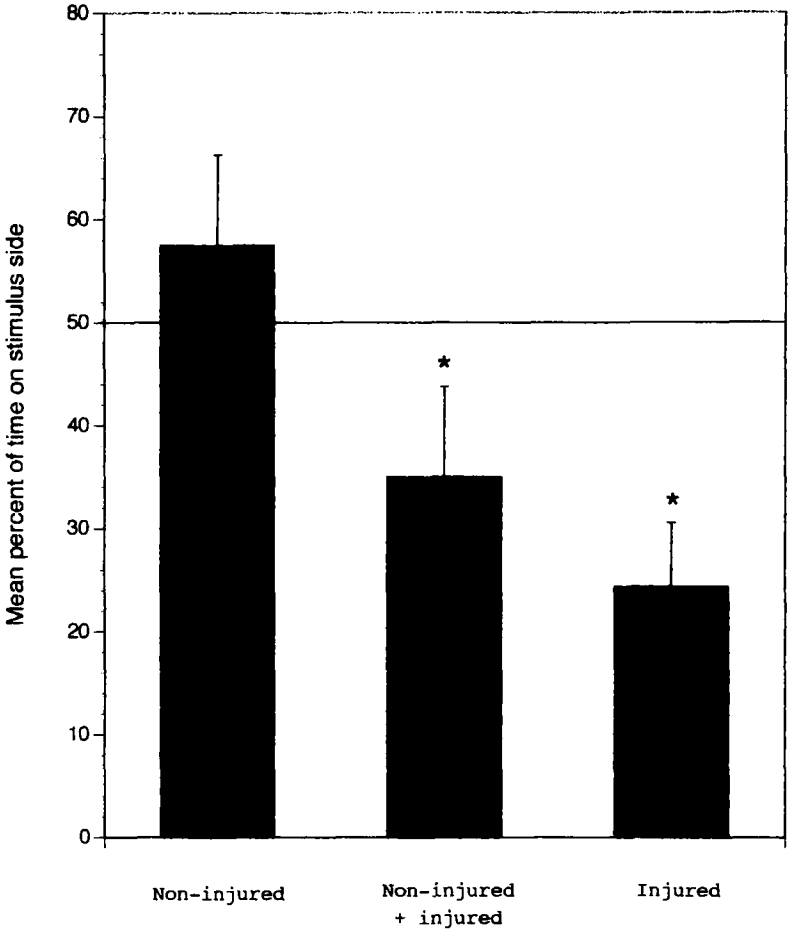

FIG. 1. Mean (+ SE) percentage of time test salamanders spent on the stimulus side of the experimental containers. Means and SE are used to illustrate trends in the data only. The data were analyzed with two-tailed binomial tests. *Significant difference at $P < 0.05$.

were as effective as chemical stimuli from injured tadpoles in eliciting alarm responses in toad tadpoles. Lutterschmidt et al. (1994) suggested that pseudotritontoxin (PTTX), a toxin contained in plethodontid salamanders, may function as the alarm cue in *Desmognathus ochrophaeus*. Ambystomatid salamanders are

known to possess toxins in their skin (e.g., Brodie and Gibson, 1969; Mason et al., 1982). As in other amphibians, these chemicals may also serve a dual function as both predator deterrents and alarm cues.

The behavioral response we quantified was avoidance of the alarm cue. This is a common response of a variety of prey to the presence of alarm cues (e.g., Lutterschmidt et al., 1994; Chivers and Smith, 1994). By avoiding an area where a predator has recently captured a conspecific, the prey should be able to lower their risk of predation by avoiding the predator's foraging microhabitat. Under natural conditions, multiple exposures of prey to alarm cues in specific microhabitats may facilitate recognition of the area as dangerous. It is unknown whether such an effect occurs in amphibians. However, some prey fishes are able to learn the identity of dangerous microhabitats based on the presence of alarm cues (Chivers and Smith, 1995; Wisenden et al., 1995a).

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FACTORS AFFECTING BEHAVIORAL PHASE TRANSITION IN THE DESERT LOCUST, *Schistocerca gregaria* (FORSKÅL) (ORTHOPTERA: ACRIDIDAE)

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Abstract—The behavior of the desert locust, *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae), is adjusted rapidly to population density and is a phase characteristic. We used discriminant analysis to quantify the extent of phase transition from the solitary to the gregarious phase and accurately classify the phase status on the basis of two decisive behavioral parameters: nymphal activity and social interaction. Fecal extracts, examined by olfactometry, attract solitarious nymphs but do not contribute to behavioral phase transition. Neither do visual stimuli alone. Short-range olfaction of airborne volatiles slightly affects behavioral phase transition. Antennectomy abolishes density response. Cuticular lipid extract, containing presumptive contact pheromones, does not attract nymphs, but does significantly affect behavioral phase transition.

Key Words—*Schistocerca gregaria*, desert locust, phase transition, behavior, cuticular lipids, contact pheromones, olfactometry, discriminant analysis.

INTRODUCTION

Locusts are in essence polymorphic grasshoppers whose morphology and behavior are affected by population density. Isolated solitary individuals are cryptically colored, tend to avoid contact, and are more subdued in activity. At high population density, gregarious chromatic forms dominate. The term "phase tran-

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sition" is used here to designate the transformation from the solitary to the gregarious phase.

In the field, dense populations evolve as a consequence of a variety of environmental and biotic factors (Rainey, 1962). Among these, seasonal winds leads to the convergence of scattered adult locusts (Rainey, 1989), while restricted local vegetation attracts and encourages the congregation of locust nymphs or adults (Kennedy, 1939). Aggregation of solitary desert locusts occurs in the field at favorable oviposition sites (Roffey and Popov, 1968). Finally, precipitation and increased quality and quantity of vegetation together promote the development of large populations, initially maintained as cohesive bands of hoppers and later as adult swarms (Uvarov, 1966, 1977).

Polymorphic changes occur gradually over several molts. In contrast, the behavioral response to changes in population density is rapid, sometimes within hours (Ellis, 1962, 1963; Gillett, 1988; Roessingh and Simpson, 1994). In broad terms, two behavioral characteristics are dominant features of phase-related behavior. Gregarious locusts are more active than are solitary locusts, and they also interact socially, whereas solitary locust do not (Uvarov, 1966).

Locusts communicate chemically in the process of phase transition. It is well known that the polymorphic and behavioral response of solitary locusts occurs when gregarious locusts are present in their vicinity (Gillett, 1968). There has been some uncertainty concerning the direct role of locust-derived volatile aggregation factors, loosely termed gregarization pheromones (Nolte, 1970, Gillett and Phillips, 1977) in phase transition. Aggregation pheromones, species-specific stimuli for convergence of both sexes, together with nonspecific attractants, contribute to an increase in population density. Cohesion pheromones maintain aggregation (Fuzeau-Braesch et al., 1988). Definitive locust phase transition implies more than aggregation and cohesion. Distinctive behavioral characteristics are expressed (Uvarov, 1966, Roessingh et al., 1993), followed much later by polymorphic changes.

In order to rapidly differentiate between these different pheromone functions, we have devised a quantitative bioassay for evaluating phase-related behavior and have concurrently measured attractancy of different locust-derived compounds by olfactometry. The contributions of the different parameters characterizing phase transition were integrated by discriminant analysis and a discriminant function was derived that defines a relative phase index. Progressive phase transition was evaluated by aligning the behavioral response to various effectors on a sliding scale, relative to the behavior of the solitary nymphs versus the behavior of nymphs from a crowded culture. Comparison of these different behavioral responses to different locust-derived compounds—attractancy on the one hand and phase-related behavior (activity and social interaction) on the other hand—indicates that aggregation and phase behavior are induced by different factors.

METHODS AND MATERIALS

Insects

The culture of the desert locust, *Schistocerca gregaria* (Forskål), used in these experiments was established from individuals field-collected in Niger in 1991. Cultures of nymphs were maintained under crowded conditions (designated gregarious) at a controlled temperature of 28 ± 2°C within the cages, under constant illumination and density of >200 locusts per cage (50 × 50 × 60 cm). They were fed fresh grass supplemented with flaked oats. First-generation nymphs were reared in isolation (designated solitary) from the middle of the second instar in small aluminum rearing cages (20 × 20 × 10 cm), partitioned by aluminum into four chambers. They were fed fresh wheat sprouts and flaked oats and kept in a temperature controlled room (28 ± 2°C), separate from the stock culture. All behavioral assays were performed with developmentally synchronized mid-fourth-instar first-generation isolated nymphs three days after the L_3 to L_4 molt.

Behavioral Assays

Ten gregarious nymphs, chosen at random from the crowded culture, or 10 solitary nymphs, were placed together in a 45-cm-diameter circular arena and videotaped for 15 min. The first 5 min were found to be necessary for adaptation of the nymphs to their unfamiliar surrounding, during which time their behavior was erratic. Behavior is stable for at least 30 min thereafter (Heifetz, unpublished). The second 5-min interval was therefore assessed for six behavioral parameters, as follows:

Social Interactions. Four parameters of the positional relations of the 10 nymphs in the arena were measured by examining three time frames with a Panasonic AG7355 video recorder. The first frame was taken 5 min after the adaptation period and the next two at 1 min and 2 min thereafter.

An associative index (AI) was calculated from the following formula:

$$AI = \sum_{i=1}^{10} \frac{n_i}{10}$$

where n_i signifies the number of nymphs positioned within one body length of the reference nymph i (each nymph in its turn).

For the number of groups, a group is defined as two or more nymphs located within one body length from one another.

The number of nymphs within the groups was determined.

For the distribution of nymphs, the distances separating each of the 10 nymphs in the arena from the other nymphs were measured from three individual

frames of the video recording, as detailed above. Analysis was performed on a Power Macintosh 7100/66AV (equipped with an LG-3 Scientific Frame Grabber, Scion Corp., Frederick, Maryland) using the public domain NIH Image program, ppc 1.56 (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, Virginia 22161, part number PB93-504868). The log variance from the average distance was calculated.

Activity. Two activity-related parameters of the 10 nymphs were measured during the 2 min following the first video frame examined for the associative index: The activity level (AL) is the mean amount of time spent in movement by each of the individual 10 nymphs in the arena. The relative rate of movement was measured by capturing 120 consecutive frames from the video cassette at 1-sec intervals and substracting one frame from another. Movement was analyzed by the NIH Image program, ppc 1.56 (see above) and expressed as the area of body displacement of the 10 nymphs per second.

Discriminant Analysis

Discriminant analysis (Dillon and Goldstein, 1984) of the behavioral parameters was performed (SAS, 1992) in order to determine the phase affiliation of the experimentally manipulated nymphs. Discriminant analysis categorizes individuals to one of K distinct groups on the basis of the values of interrelated normal variables ($X_1, \ldots, X_p$). A linear combination ($Y = a_1X_1 + \cdots + a_pX_p$) of the normal variables is sought, using data for individuals of known affiliation, which exhibits maximal differences between groups, relative to differences within groups. In this present case, a discriminant function Y was then derived to determine the phase index of individuals for which values of $x_1, \ldots, x_p$ are available but whose group affiliation is unknown.

The classification of nymphal behavior is presented as a phase index on a sliding scale, calculated from a discriminant function. Negative values are classified as solitary and positive values are classified as gregarious. The discriminant function is calculated from the distinctive behavior of nymphs conditioned to either isolation or crowding. Percentage phase prediction indicates the degree to which the replicate bioassays conform to the predicted phase category (i.e., the average phase index may position the behavior on the solitary side of the threshold of discrimination, while a certain percentage of the replicates are individually categorized by the discriminant function as gregarious, and vice versa).

Evaluation of Isolated Sensory Stimuli.

Nymphs of the gregarious stock culture were isolated from the second instar and kept in individual chambers of a small aluminum cage (20 × 20 × 10 cm),

partitioned into four separate chambers by either aluminum, glass, or wire screening until mid-fourth instar. A fourth group consisted of four nymphs reared together from the second instar in a cage of similar dimensions but with no partitions. The discriminant function was derived from the behavior of the group of four nymphs and that of the solitary nymphs.

Collection of Airborne Volatiles

This was adapted from Yarden and Shani (1994). Volatiles were collected at different times in 3-hr intervals from groups of 60 fourth instar nymphs placed in a 3-liter flask connected to an all-glass system (see Figure 1) and subjected to a continuous flow (15–19 ml/min) of compressed clean dry air (Maxima, Ramat Gan Israel) and further purified by passing through a trap of silica blue (Merck Co., Darmstadt, Germany) and Porapak Q (Millipore Co., Millford, Massachusetts) filters. The outlet tube from the flask led into a trap of hexane cooled in ice and acetone. The hexane solutions were later reduced in volume by a gentle flow of N_2 gas and used for the behavioral assays.

Synthetic Phenol Derivatives

Several candidate compounds previously identified in air collected from locust colonies (Fuzeau-Braesch et al., 1988) were purchased from Sigma and individually dissolved in chloroform.

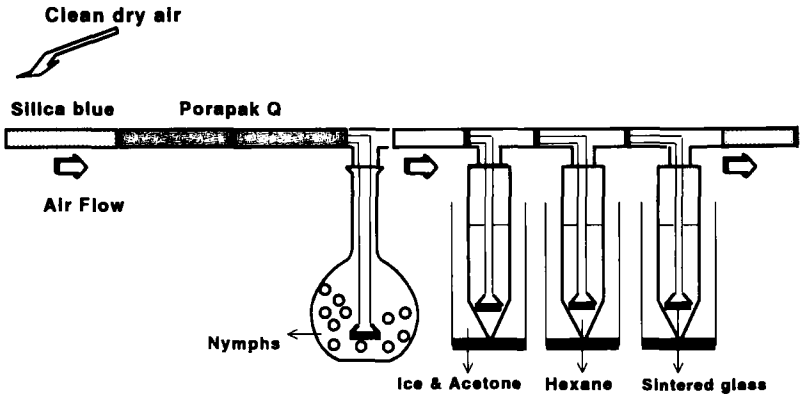


FIG. 1. Diagram of extraction apparatus for collection of airborne volatiles from crowded fourth instar nymphs of the desert locust. Groups of 60 nymphs were placed in a 3-liter glass flask and volatiles were entrained in a stream of clean air and captured in a series of cold traps.

Cuticular Surface Extracts

Groups of 50 nymphs were taken from the crowded culture, immersed in 150–200 ml of dichloromethane and gently shaken for 10 min. The extracts were collected and reduced in volume.

Fecal Extracts

Cages of crowded nymphs were cleaned of feces every 5 hr, and the collected material weighed and stored at −20°C until extracted with dichloromethane, as above. The dichlorometane extracts were reduced in volume in a rotary vacuum evaporation system (Rotavapor-R, Büchi, Switzerland).

Treatment of Solitary Nymphs

Nymphal volatiles were applied to Whatman No. 1 filter paper disks that were impregnated with paraffin oil to attenuate the rate of release. Cuticular extracts, feces extracts, or synthetic phenol derivatives were applied to 10 × 15-cm polyethylene screens as solutions in organic solvents. The solvents were evaporated and one disk or screen placed in each chamber. Solitarious nymphs were exposed to treated or control screens (pure solvent only applied) for 5 hr. Ten exposed nymphs of each treatment were then placed together in the arena and their behavior assayed.

Olfactometry

A Y-shaped glass olfactometer system was designed with an inner diameter of 2.5 cm. The outlet tube was 20 cm in length and each of the two arms were 18 cm in length. These dimensions are sufficient for a locust nymph to move through comfortably. The connections of the glass Y-shaped olfactometer to other components of the system were with S-40 ground glass connectors. Each arm led to a glass trap and from there to a flask. One flask contained a test preparation, and the other remained empty. The olfactometer was attached at its outlet to a vacuum pump (ABM, Germany) and a constant flow rate of 60 ml/min was maintained and monitored. A second flowmeter was attached with Tygon tubing via a cylinder of activated charcoal to the inlet of each flask in order to equalize the airflow rate through the control and test flasks. Individual nymphs were released at the beginning of the outlet tube and monitored as they moved against the airflow into either of the two arms of the olfactometer, after which they fell into the trap. Extracts of nymphal cuticle, feces, and airborne volatiles were assayed separately, and their attraction for single solitarious nymphs was assessed.

RESULTS

Solitary and Gregarious Behavior

Six parameters were evaluated for their contribution to the discrimination between gregarious and solitary nymphs. They were all found to be significantly affected by rearing density (Table 1) with univariate t tests. Correlation analysis and stepwise discriminant analysis (SAS 1992) detected various degrees of correlation between the different variables. The most striking differences were observed between activity levels and associative indices: Gregarious nymphs are almost three times as active as solitary nymphs ($P < 0.0001$), and the associative index of gregarious nymphs is about double that of solitary nymphs ($P < 0.0003$). These two parameters were chosen by stepwise discriminant analysis as sufficient for discriminating between the two phases, and the discriminant function was derived from them only.

Effect of Density

Behavior was evaluated by placing nymphs together in groups of 2–10 from the second until mid-fourth instar. Phase transition is considerably shifted in groups of three nymphs together and crosses the threshold for discrimination of gregarious phase in groups of five nymphs (Figure 2). Crowding 10 nymphs together induces even more marked gregarious behavior, although less than that elicited with highly crowded nymphs from the standard culture. In addition to the shift in phase index, which approaches and then crosses over the threshold for discrimination of gregarious behavior, the percentage of nymphs classified as gregarious increases.

TABLE 1. PARAMETERS OF ACTIVITY AND SOCIAL INTERACTIONS DESCRIPTIVE OF GREGARIOUS AND SOLITARY DESERT LOCUST FOURTH-INSTAR BEHAVIOR[a]

	Activity level[b]	Associative index	Groups (N)[c]	Nymphs (per group)	Rate of movement[d]	Distribution[e]
Solitary	24.15 ± 3.3	0.3 ± 0.05	1.08 ± 0.1	2.13 ± 0.2	3.20 ± 0.8	2.10 ± 0.03
Gregarious	69.52 ± 3.4	0.77 ± 0.09	1.93 ± 0.1	2.93 ± 0.2	6.05 ± 0.4	2.23 ± 0.02
Significance (P)	<0.0001	<0.0003	<0.0001	<0.0128	<0.0069	<0.0203

[a] Average of 10 replicates ± SEM.
[b] Number of seconds spent in activity during 2 min.
[c] Individual nymphs are regarded as associated in a group if they are within one body-length of any other nymph.
[d] Area of body displacement during 2 min.
[e] Log variance of distribution of the 10 individual nymphs within the arena.

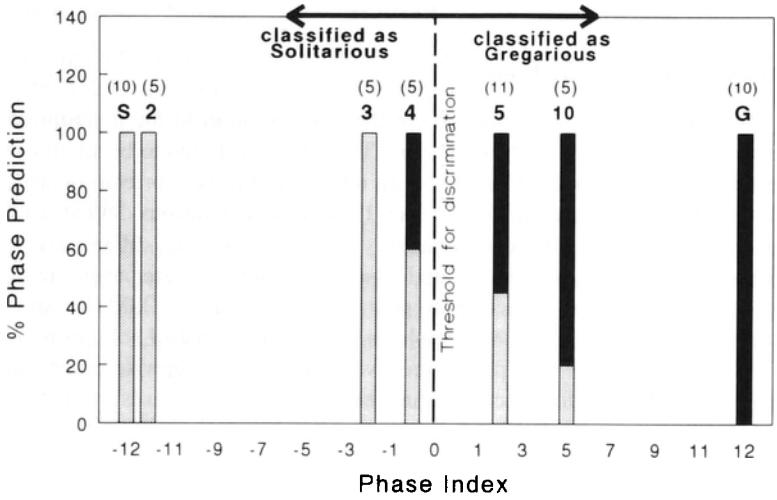


FIG. 2. Phase indices (PI) of desert locust nymphs reared at different densities in chambers measuring 10 × 10 × 10 cm from second to mid-fourth instar. The bold letters and numerals above the bars indicate density: **S** = 1 nymph per chamber (solitarious), **2–10** = number of nymphs per chamber; **G** = nymphs from standard culture (200 per large cage). ▬ = percentage gregarious behavior; ▬ = percentage solitary behavior. Numbers in parentheses are numbers of replicate experiments at each density. PI was calculated from data of solitary and high-density standard culture nymphs (PI = 0.45 AL + 9.14 AI − 25.78).

Asymetrical Plasticity of Phase Behavior

Newly eclosed fourth instars from the crowded culture were isolated for three days and their behavior assayed. When regrouped 10 together, reversal to gregarious behavior was evident after 6 hr, and behavior completely reverted to gregarious behavior after the nymphs were regrouped for 24 hr. The change of gregarious to solitary nymphal behavior is less rapid than the converse. When newly eclosed fourth instar nymphs were kept at a density of 10 per cage for three days and then isolated, only partial behavioral changes were evident 24 hr later when 45% of the groups were still behaving gregariously (Figure 3).

On the basis of the effects of density and time, an exposure time of 5 hr of solitary nymphs to various chemical affectors was routinely chosen, and the discriminant functions were calculated according to the nymphal equivalents of compounds assessed in separate experiments.

A variety of components—visual recognition at short-range, sensillar chemoreception, and/or tactile mechanoreception—are presumably involved in the

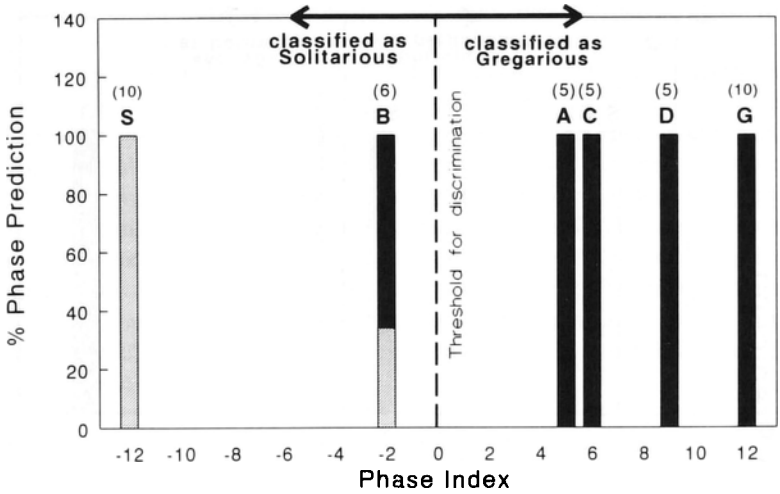


FIG. 3. Phase indices of desert locust nymphs of standard culture isolated for three days and then regrouped for 6 hr (**C**) or 24 hr (**D**) or distributed at a density of 10 nymphs per chamber for three days, and then separated for 6 hr (**A**) or 24 hr (**B**). **S** = solitary; **G** = 10 nymphs crowded for 4 days.) ■ = percentage gregarious behavior; ■ = percentage solitarious behavior. Numbers in parentheses are numbers of replicate experiments of each treatment. PI was calculated from data of solitarious nymphs and of the density of 10 nymphs (PI = 0.44 AL + 25.91 AI − 30.12).

induction of phase transition. We evaluated the relative contribution of these different stimuli to the process of phase transition by assaying each component separately.

Effect of Sensory Stimuli on Phase Transition. Solitary nymphs were obtained by rearing them individually in aluminum-partitioned cages, thereby isolating them from all interactive stimuli. Visual stimuli alone do not shift the phase index towards gregariousness: this was demonstrated by replacing the aluminum partitions with glass, thus depriving them of olfactory and tactile stimuli. When separated by wire screening only (i.e., deprived of tactile stimuli but exposed to olfactory and to somewhat restricted visual stimuli, 33% behaved gregariously (Figure 4a).

Antennal chemoreceptive sensilla are the main sensors for olfactory stimuli (Blaney and Simmonds, 1990). In order to evaluate whether phase transition occurs in the absence of antennae, the behavioral response of antennectomized crowded nymphs was examined.

Antennal Sensory Perception. Gregarious nymphs were operated on when in the fourth instar and kept together for an additional 24 hr after surgery. In

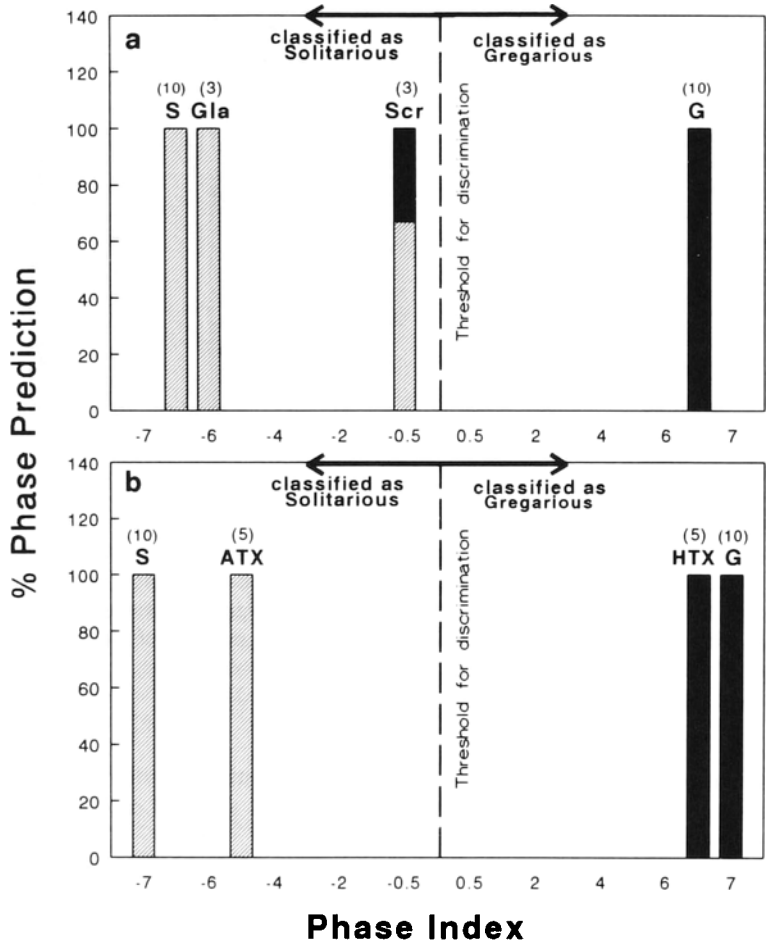


FIG. 4. Phase indices of (a) effects of visual, olfactory, and contact stimuli on phase transition. Nymphs were reared from second to mid-fourth instar in chambers separated by partition. **S** = aluminum partition (solitarious); **Gla** = glass partition; **Scr** = wire screen partition; **G** = group of four nymphs together with no partition. PI was calculated from data of solitary nymphs and of the density of four nymphs in a 20 × 20 × 10 cm cage (PI = 0.28 AL + 11.13 AI − 15.93). (b) Effects of antennectomy (**ATX**) or hind-tarsal amputation (**HTX**) of mid-fourth-instar nymphs from the standard culture, kept together in groups of four for an additional 24 hr. PI was calculated from data of solitary nymphs and of the density of four nymphs in a 10 × 10 × 10 cm cage. ▬ = percentage gregarious behavior; ▬ = percentage solitarious behavior. Numbers in parentheses are number of replicates. (PI = 0.36 AL + 17.32 AI − 21.23.)

the absence of sensory input from the antennae, the crowded nymphs were behaviorally solitary, and neither tactile interactions nor vision was able to maintain gregarious behavior (Figure 4b). Hind tarsal amputation was performed on separate groups as a control surgical procedure, but the surgery was unable to depress the interactive stimuli maintaining gregariousness. The solitary behavior did not result from a reaction to surgery, but rather from sensory deprivation.

Experiments were designed to identify the character of the presumptive chemical stimuli essential for behavioral phase transition. Different experimental treatments were performed with material applied at doses adjusted to different nymphal equivalents. A nymphal equivalent was defined as the amount of material derived from one nymph. Standardization of the phase index was achieved by deriving different discriminant functions from the behavioral data of specific nymphal densities.

Effect of Locust-Derived Compounds on Induction of Gregarious Phase Behavior.

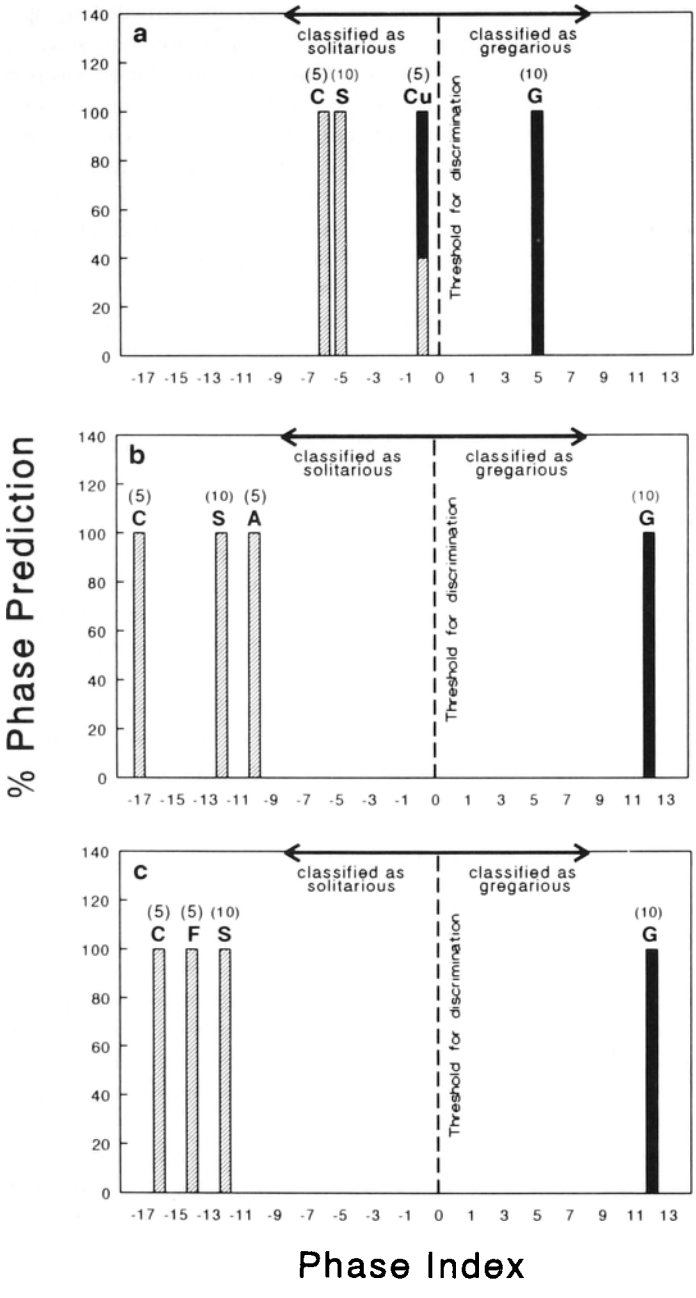
Exposure of solitarious nymphs for 5 hr to extracts of nymphal cuticle (5 nymphal equivalents) strongly shifted the phase index, and 60% of the groups behaved gregariously in the behavioral test (Figure 5a). Exposure to airborne nymphal volatiles (10 nymphal equivalents) had a slight effect on phase index (Figure 5b), while extract of nymphal feces (4 g/ml, or 50 nymphal equivalents) had none (Figure 5c).

Airborne phenol derivatives such as guaiacol and veratrole (Fuzeau-Braesch et al., 1988) are present in fecal extracts (Torto et al., 1994; Obeng-Ofori et al., 1994). Individual and combined effects of phenols on nymphal behavior was next assessed.

Behavioral Response to Phenol Derivatives

Solitary nymphs were exposed for 5 hr to 5 mmol guaiacol, veratrole, or both veratrole and guaiacol together. These two compounds, particularly guaiacol, slightly affected activity levels (data not shown). We tend to attribute this slight increase in activity to nonspecific sensory stimulation of nymphal chemoreceptors. Associative indices were not significantly affected by treatment with these odorants either separately or together (data not shown). When subject to discriminant analysis, veratrole or guaiacol alone, or both together, did not affect phase index (Figure 6).

Cuticle extracts markedly shifted the phase index of exposed solitary nymphs toward the gregarious state. They might conceivably have been acting indirectly as attractants, leading to aggregation, whereas other short-range factors might thereafter induce gregarious phase behavior. Attraction was therefore next examined in an olfactometer.


a
classified as solitarious
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C S Cu G
Threshold for discrimination
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c
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Phase Index

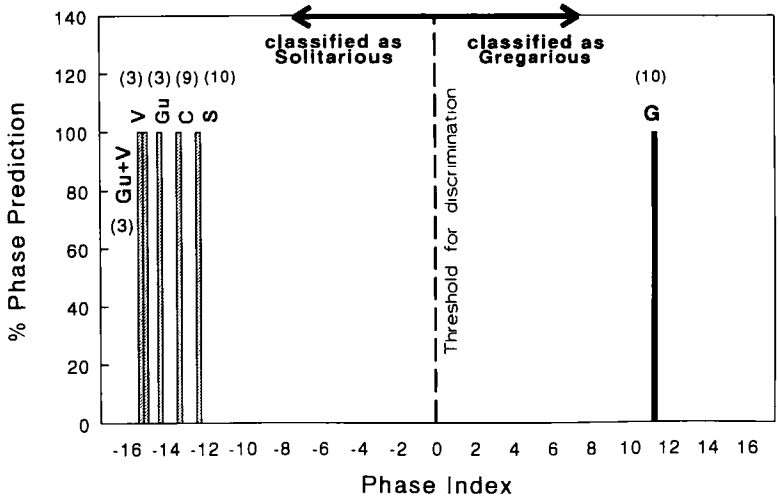

FIG. 6. Phase indices of solitary nymphs after they were exposed in individual chambers for 5 hr to 5 mmol guaiacol (**Gu**); veratrole (**V**); or both together (**Gu + V**). **S** = solitary; **G** = gregarious; **C** = control (screen treated with chloroform). PI was calculated from data of solitary and gregarious nymphs. Numbers in parentheses are number of replicates. (PI = 0.45 AL + 9.14 AI − 25.78.)

Olfactometry

No significant attraction of solitary nymphs to cuticular extracts was observed in olfactory assays under conditions where no contact could occur between the extracts and the test nymphs (Figure 7). In contrast, orientation in the olfactometer of female and male nymphs to extracts of nymphal feces was two-fold higher than nonspecific movement into a flow of clean air (χ^2 = 12.73 and 18.57, respectively; $P < 0.001$).

FIG. 5. Phase indices of solitarious nymphs after they were exposed in individual chambers for 5 hr to (a) 5 nymphal equivalents of cuticle extracts (**Cu**) (PI = 0.28 AL + 5.02 AI − 12.66); (b) 10 nymphal equivalents of airborne volatiles (**A**) (PI = 0.44 AL + 25.91 AI − 30.12); or (c) 50 nymphal equivalents of fecal extracts (**F**) (PI = 0.45 AL + 9.14 AI − 25.78). **S** = solitary, **G** = crowded at different densities (a, 5 nymphs per chamber; b, 10 nymphs per chamber; c, >200 nymphs per cage); **C** = control (screen treated with solvents). ■ = percentage gregarious behavior; ■ = percentage solitary behavior. Numbers in parentheses are number of replicates.

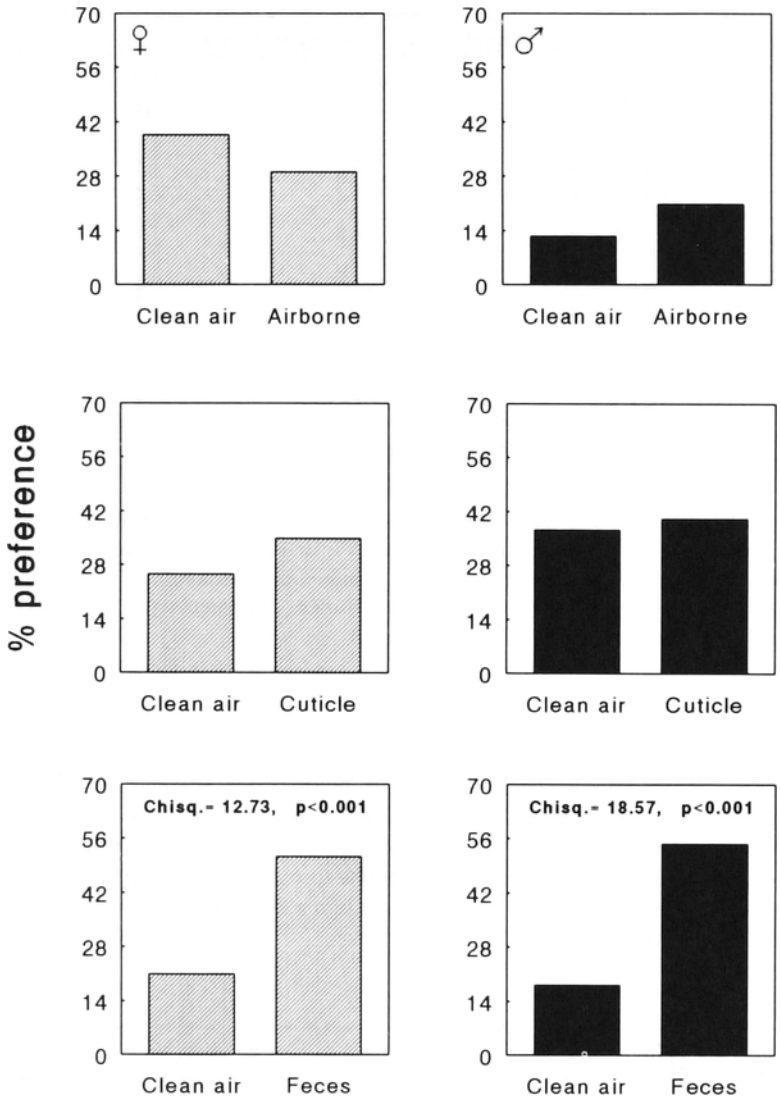

FIG. 7. Attractancy of locust-derived airborne volatiles, cuticle extracts, and fecal extracts (same concentrations as in Figure 5) to individual solitary female and male nymphs, in a Y olfactometer. $N = 50$.

DISCUSSION

Locust phase behavior is a rapid response to changes in population density. The relative importance of the various behavioral phase characteristics and the degree of their expression at different population densities have not been sufficiently evaluated. A quantitative bioassay of sufficient sensitivity to measure partial phase transition was designed and evaluated by discriminant analysis (Dillon and Goldstein, 1984). Stepwise discriminant analysis independently chose and limited observations and measurements of behavior to those essential for describing the phase status of locust nymphs that had been exposed to various treatments. Activity levels and associative indices were found to be essential and sufficient to attributing the experimental group of locusts to either of the two phases. The merit of discriminant analysis is that it can assist in critically evaluating partial effects and quantifying the intensity of response on a sliding scale of phase index.

Density-related behavioral response is not an all-or-nothing event, and moderate crowding elicits intermediate behavior (Uvarov, 1966). In the experimental design used in these present experiments, a marked shift in behavior at a density of three nymphs was observed, while the behavior at a density of five nymphs was unequivocally classified as gregarious. Gregarious behavior of 10 nymphs crowded together was even more pronounced but still distinguishable in intensity from that of nymphs of the standard locust colony. The intensity of response is density-dependent.

The duration of exposure to the different densities or treatments affects the intensity of phase shift as well. Six hours were required for nymphs reared in isolation to behave gregariously when placed 10 together, and after 24 hr of regrouping they were classified as markedly gregarious. However, 6 hr of isolation only slightly shifted the phase index of gregarious nymphs towards solitary behavior, while 24 hr of isolation were required for them to cross the threshold to solitary behavior. After being separated for four days during the fourth instar, the phase index approaches that of solitarious nymphs. These results are consistent with previous reports that transition to gregarious behavior can occur within 0.5–4 hr of crowding, whereas transition from crowded to solitary nymphal behavior is much slower (Ellis, 1962, 1963; Gillett, 1988; Roessingh and Simpson, 1994).

Visual, olfactory, and tactile stimuli are reported to be essential for gregarious phase induction (Gillett, 1968). We reexamined individual roles and found that visual stimuli did not induce behavioral phase transition, while olfaction (presumably antennal) shifted the phase index considerably. This is corroborated by the observation that when gregarious nymphs were antennectomized and kept together (thus with lack of antennal chemoreception but maintenance

of visual stimuli), solitary behavior was induced. Hind-tarsal amputation did not elicit this response. We conclude that lacking antennal chemoreception, gregarious behavior cannot be maintained even under crowded conditions. This complements observations of chromatic changes in desert locust nymphs after antennectomy (Mordue, 1977), interpreted as due to reduction in sensory input, and to behavior indicative of the solitary phase in crowded nymphs after antennectomy, but not after tarsal amputation (Gillett, 1983).

Various volatile degradation products of plant-derived lignin from fecal extracts have been studied in the context of locust gregarization. One of these, 5-ethyl guaiacol, was reported to reduce the rate of melanin loss by isolated nymphs (Nolte, 1963) and to affect chiasma frequency (Nolte, 1967), but its bioactivity and the validity of its identification were subsequently questioned (Dearn, 1974; Fuzeau-Braesch *et al.*, 1988). We found that fecal extracts did attract individual solitary nymphs, but that gregarious phase behavior was not induced. These results are in accord with previous reports demonstrating that group formation is not promoted by chronic exposure of isolated nymphs to feces or fecal extracts (Gillett, 1983).

The attractant effects of phenol, guaiacol, and other odorants from culture cages derived directly from the locust and presumably also from the feces have been previously examined with locust nymphs and adults (Fuzeau-Braesch et al., 1988). In our experiments, we did not find any effect of guaiacol and veratrole, or both together, on phase transition, nor did total airborne volatiles attract solitary nymphs. However, airborne volatiles did shift phase behavior somewhat.

The most consistent effects on behavioral phase index were elicited by cuticular components. Cuticular components did not attract solitary nymphs, and this is in contrast to previous studies that had suggested that cuticular components were involved in mutual attraction between locusts (Norris, 1963, 1964). Presumably, complete phase transition is induced by a variety of stimuli together. Each might also be expected to elicit only a partial response when acting alone. The shift in the phase index was somewhat less than when nymphs were in contact, but this might also have been due to the marginal concentration applied.

All of these results lead to the conclusion that a fundamental difference exists between attraction (i.e., effect of fecal extracts) and phase-related behavior (i.e., effect of cuticular extracts). Fecal extracts strongly attracted fourth instar nymphs but did not affect the phase index, while the cuticular extract did not attract at all, but did shift behavioral phase.

We find that these controlled laboratory bioassays relate closely to events occurring in the field, where a series of sequential events presumably induces the gregarious phase. Wind convergence, aggregation on available vegetation, and particularly sexual attraction, and attraction to limited oviposition sites bring solitary adults together. Initial aggregation may be enhanced by feces-derived

attractant compounds. Visual stimuli are not necessarily essential for these initial events but could later contribute to recognition of the gregarious phase and cohesion of hopper bands. It is the mutual contact of nymphs, aggregated by environmental factors or locust-derived chemicals, that finally leads to the induction of behavioral phase transition, presumably mediated by cuticular contact pheromones.

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ELECTROANTENNOGRAM RESPONSES OF *Hyles lineata* (SPHINGIDAE: LEPIDOPTERA) TO VOLATILE COMPOUNDS FROM *Clarkia breweri* (ONAGRACEAE) AND OTHER MOTH-POLLINATED FLOWERS

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Abstract—Electroantennograms (EAGs) from field-collected *Hyles lineata* moths were recorded in response to 10 individual floral volatiles identified from *Clarkia breweri* (Onagraceae), to 22 scent compounds produced by other moth-pollinated flowers and to eight ubiquitous "green leaf volatiles." Females' EAGs were generally 1.5- to 2-fold greater than those observed for male moths. Female : male EAG rank orders were significantly correlated, but marked differences in order were observed for some compounds (e.g., benzyl alcohol, cinnamic aldehyde, geraniol, and linalool). Linalool, benzyl acetate, methyl salicylate, and pyranoid linalool oxide elicited the largest EAG responses (−1.2 to −0.8 mV) among scent compounds from *C. breweri*. EAG responses were significantly lower for monoterpenes as a pooled compound class than for aromatic esters, alcohols and aldehydes, fatty acid derivatives, N-bearing compounds and oxygenated terpenoids. EAG responses to structurally related scent compounds were not significantly different in most

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cases. Both male and female *H. lineata* were sensitive to most *C. breweri* scent compounds at 10^{-2} to 10^{-4} $\mu g/\mu l$ doses, and rank order in potency varied with the dose/concentration tested. *H. lineata's* olfactory sensitivity to diverse volatile compounds across a range of doses/concentrations suggests that a broad array of volatiles could function as floral attractants for foraging hawkmoths.

Key Words—Aromatics, EAG, floral attraction, floral scent, green leaf volatiles, hawkmoths, monoterpenes, nitrogen-bearing compounds, olfaction, semiochemicals, *Hyles lineata*.

INTRODUCTION

The white-lined sphinx moth, *Hyles lineata* L. (Sphingidae: Lepidoptera), is the most widely distributed hawkmoth species in the world; it is present throughout the Americas (subsp. *lineata*), across Europe, Asia, and Africa (subsp. *livornica*) and in Australia (subsp. *livonicoides*; Rothschild and Jordan, 1903; von Knoll, 1925; Hodges, 1971). *H. lineata* moths are avid flower visitors, foraging for nectar at dusk and early evening in most localities (Clements and Long, 1923; Gregory, 1963, 1964; Kislev et al., 1972; Hodges, 1995). Due to its general abundance and broad distribution, *H. lineata* is probably the most important hawkmoth pollinator throughout much of North America (Cruden, 1970; Stockhouse, 1973; Chase and Raven, 1975; Miller, 1978, 1981). The broad acceptance of flower types by *H. lineata* throughout its distribution suggests either that it is attracted to a wide variety of floral scent compounds (or blends thereof) or that it relies on cues other than floral scent (i.e. visual cues) during foraging. One way to test these possibilities is to study the physiological and behavioral responses of *H. lineata* to the scent compounds of the flowers that it visits.

H. lineata moths visit a broad spectrum of plant species bearing flowers of diverse morphological classes (von Knoll, 1925; Fleming, 1970; Kislev et al., 1972). Grant (1983, 1985) cited *H. lineata* as a visitor to flowers of 41 native plant species from 13 angiosperm families in North America. However, hawkmoths are observed to be effective pollinators of a subset of these plants, those with tubular or trumpet-shaped flowers in which the nectar tubes or spurs are as long as or longer than the extended proboscides of the moth. This geometrical relationship increases the probability of head/body contact with floral sex organs, leading to removal and deposit of pollen (Gregory, 1964; Miller, 1978; Grant, 1983; Dafni et al., 1987; Nilsson, 1988; Hodges, 1995; Raguso, 1995). Many hawkmoth pollinated flowers often are pale in coloration, open during the evening, produce copious amounts of nectar, and emit a strong, sweet aroma. This combination of floral traits has evolved repeatedly in most major angiosperm families worldwide and defines the "hawkmoth-pollination syndrome" (Baker,

1961; van der Pijl, 1961; Gregory, 1963; Faegri and van der Pijl, 1979; Miller, 1981; Grant, 1983).

In western North America, *H. lineata* pollinates a number of plant species with pale, tubular, scented flowers, especially within the genera *Aquilegia* (Ranunculaceae), *Mirabilis* (Nyctaginaceae), and *Oenothera* (Onagraceae; reviewed by Grant, 1983, 1985). One plant species typical of this assemblage is *Clarkia breweri* (Gray [Greene]: Onagraceae). *H. lineata* and other hawkmoths are important pollinators of *C. breweri* in the central Coast Range Mountains of California (Raguso, 1995). Genetic and morphological data suggest a recent origin of *C. breweri* from scentless, bee-pollinated ancestors (Lewis and Lewis, 1955; MacSwain et al., 1973; Gottlieb and Weeden, 1979; Raven, 1979). Moth pollination and strong floral scent emission are not observed in any other *Clarkia* species, and are thought to represent a derived condition in the genus (MacSwain et al., 1973; Raven, 1979; Raguso and Pichersky, 1995). The currently accepted model of hawkmoth foraging behavior proposes that floral scent functions as a sign stimulus that "turns on" or releases anemotactic searching flight behavior, followed by visual close-range orientation to flowers (Baerends, 1950; Brantjes, 1973, 1978). Following this model, we propose that floral scent has evolved in *C. breweri* as an adaptive trait modified through selective pressure by hawkmoth pollinators, including *H. lineata* (Raguso and Pichersky, 1995). For this hypothesis to be valid, hawkmoths must be olfactorily sensitive to the array of *C. breweri's* floral scent compounds and able to detect them at concentrations low enough to serve as cues for flower location from a distance.

We tested the olfactory sensitivity of *H. lineata* moths by measuring their electroantennogram (EAG) responses to the floral scent compounds of *C. breweri*. EAGs have been used to assess the olfactory sensitivities of agriculturally important moth species in response to host plant volatiles (Grant, 1971; Adler and Jacobson, 1972; Tichenor and Seigler, 1980; Gabel et al., 1992; Light et al., 1993; Zhu et al., 1993) and to sex pheromones (Schweitzer et al., 1976; Reed et al., 1987). Little is known, however, about the breadth of hawkmoths' olfactory responses to floral volatiles from different chemical structural classes, or to plant vegetative volatiles in general. The purpose of our study was to characterize, through EAG recordings, the peripheral olfactory responses of *Hyles lineata* moths to 40 scent compounds, including ten floral volatiles from *Clarkia breweri*, 22 floral volatiles identified from other moth-pollinated plants and eight "green leaf volatiles" (GLVs) typical of vegetation.

We used EAG response data to address the following questions.

(1) Do *H. lineata* moths respond differently to diverse chemical classes of plant volatiles?

(2) Can *H. lineata* moths distinguish between structurally similar scent compounds bearing slightly different functional groups?

(3) Can *H. lineata* moths detect floral scent compounds at low doses?

Selective differences in olfactory response to specific compounds or structural classes of compounds might predispose *H. lineata* to respond to certain olfactory stimuli through induction of flight orientation and feeding or ovipositional behaviors. By testing *H. lineata's* responses to volatile compounds from different structural classes (terpenoids, aromatics, fatty acid derivatives, and nitrogen-bearing compounds), we can begin to characterize the potential role of olfaction in the foraging behavior of a widespread hawkmoth species.

METHODS AND MATERIALS

Insects

Adult *H. lineata* moths were collected at UV light traps from September 17 to October 4, 1993 in Tucson, Pima Co., AZ and transported live via overnight courier to Albany, CA. Upon arrival, moths were fed a 10% sucrose solution and held in a 1 m^3 screen cage within the laboratory (25°C, 12 L:12 D). Moths had free access to sucrose solution feeding stations, and were fed manually once daily.

Test Compound Justification

Appendix 1 lists the 40 scent compounds tested, their purities, supply sources, and rationale for inclusion in this study. We measured EAG responses to ten of the 12 principal volatiles identified from the floral headspace of *C. breweri*, constituting 97.9% of total volatile emissions analyzed via GC-MS (Raguso and Pichersky, 1995). *C. breweri* floral volatiles fell into two chemical classes, aromatics and oxygenated monoterpenoids, and included benzyl acetate, linalool, and methyl salicylate, three of the most prevalent floral scent components in hawkmoth-pollinated flowers (Brantjes, 1978; Kaiser, 1993; Knudsen and Tollsten, 1993).

We tested 22 additional floral volatiles from diverse chemical classes, including aromatics, fatty-acid derivatives, monoterpenes, nitrogen-containing compounds, and a sesquiterpene alcohol. Test compounds in this grouping were chosen for one of three reasons; (1) they frequently occur as floral scent constituents in many other hawkmoth-pollinated flowers (e.g., amyl salicylate, farnesol, geraniol, indole; Morgan and Lyon, 1928; Kaiser 1991, 1993; Knudsen and Tollsten, 1993), (2) they stimulate upwind flight in another family of flower-visiting moths, the Noctuidae (e.g., phenylacetaldehyde, 2-phenylethanol; Jacobson et al., 1976; Haynes et al., 1991; Heath et al., 1992), or (3) they share a common carbon skeleton with one of the above compounds, but differ by one functional group (e.g., methyl anthranylate, methyl-2-methoxybenzoate). Inclusion of these compounds allowed us to determine (1) the chemical breadth

of *H. lineata's* olfactory "vocabulary," (2) whether these moths can detect flowers that are attractive to noctuid moths, and (3) whether they can distinguish between similar odorants bearing subtle chemical differences.

We analyzed EAG responses to eight green leaf volatiles (GLVs). These C_6 and C_8 aliphatic alcohols, aldehydes, and acetates are ubiquitous in plant vegetation and provide strong structural contrast to the aromatic and terpenoid floral volatiles. Female *H. lineata* may respond behaviorally to GLVs during hostplant orientation and oviposition. In addition, these compounds are known to synergize male responses to female sex pheromone in noctuid moths (*Helicoverpa zea*, *Heliothis virescens*; Dickens et al., 1993; Light et al., 1993), and may be important to male sphingids as well.

Olfactory Stimuli

Test compounds were dissolved in HPLC-grade hexane to form 10% volumetric solutions. Compounds poorly soluble in hexane, such as indole and vanillin, were dissolved by weight in diethyl ether. One ul of each solution (approx. 100 ug of test odorant) was pipetted onto filter paper strips, which were allowed to evaporate for ca. 30 sec, then inserted into Pasteur pipettes and stored at 5°C until used. Each test cartridge was loaded 20 min prior to its presentation to the moth's antennae.

EAG Technique

EAG deflections were recorded and measured on a Tektronix 5113 storage oscilloscope as described by Light et al. (1988). Silver-chloride electrodes were prepared in drawn glass capillary tubes, and were electrolytically balanced (Raynauld and Laviolette, 1987). Living moths were mounted on a plexiglass block with a central trough molded with soft paraffin to fit the contours of the moth's ventrum. The terminal five segments of the right antenna (out of 58–61 total segments) were excised and the recording electrode was inserted into the antennal cavity, while the ground electrode was inserted into the head at the base of the antenna. The antenna was bathed continuously by a stream of charcoal-filtered, humidified air at a flow rate of one L/min. A "puff" of test compound was delivered onto the antenna when a three-way solenoid valve was activated, diverting air through the test cartridge for a 1 sec stimulation interval. Each compound stimulation was followed by a minimum 60 sec purge period of filtered air to ensure recovery of antennal receptors.

For each compound tested, EAGs were recorded from five male and seven female *H. lineata* moths. In addition, EAG responses to serial dilutions of eight floral volatiles from *C. breweri* (ranging in dose from ca. 10^{-4} to 10^{2} μg/μl per filter paper) were recorded from three males and six females. "Control" stimuli (1 μl of hexane solvent per filter paper) and "standard" stimuli (1 μl of 1%

linalool in hexane per filter paper) were interspersed about every fifth compound or dose tested. The order of compound presentation was randomized for each individual moth. Experiments ranged from 65–110 min in length; standard stimulus EAG data were plotted vs. time, and no consistent patterns were observed. All experiments involved the testing of single compounds; no blends were used.

Treatment of EAG Data

EAG responses (−mV) to test compounds were adjusted to compensate for solvent and/or mechanoreceptive artifacts by subtracting the accompanying "control" stimulation, yielding "corrected −mV" (see Reed et al., 1987; Gabel et al., 1992). EAG data also were expressed as percent responses of the standard stimulant (linalool, a compound of medium MW and volatility), hereafter written "% of standard" (see Light et al., 1992a, 1992b; van Loon et al., 1992). Due to differences in volatility between the 40 stimulant compounds (MW and boiling point data, Appendix 1), we utilized the standard stimulus as a reference point for comparing EAG responses to different stimulant compounds. Thus, "% of standard" EAG data were used in male-female rank order comparisons and all comparisons involving different chemical classes, isomers and functional groups.

EAG Data Analyses and Statistical Tests

Mean EAG responses (% of standard) to all 40 stimulant compounds (at 10% concentration) were ranked 1–40 in descending order of magnitude for both sexes. We compared male and female EAG rank orders using Spearman's Rank Correlation Coefficient (Sokol and Rohlf, 1981), with which we tested the null hypothesis of no correlation (Rho = 0) between male and female scent compound ranks. If a calculated sample product-moment correlation coefficient (R_s) was found to exceed the upper 95% confidence interval, we rejected the hypothesis of no correlation between male and female EAG rank orders.

We attempted to identify variation in EAGs associated with sex and/or differences between and within chemical classes of olfactory stimulants. EAG data (% of standard) were square-root transformed to compensate for unequal variances and were analyzed using Repeated Measures ANOVA models (Systat 5.2.1; 1992). First, we grouped floral scent and vegetative compounds by chemical class (monoterpenes, oxygenated terpenoids, aromatic esters, aromatic alcohols and aldehydes, fatty acid derivatives, and nitrogen-bearing compounds) and derived a mean response value for each chemical class by averaging the mean EAGs for the individual member compounds. These derived data were analyzed for significant differences related to sex, chemical class, and sex x class interactions using Repeated Measures ANOVA. If significant variation were asso-

ciated with chemical class, specific pairs of stimulant classes were then compared in three *a priori* contrasts: monoterpenes vs. oxygenated terpenoids, aromatic esters vs. aromatic alcohols and aldehydes, and all terpenoids vs. all aromatics. Contrasts were examined by computing Scheffé contrast intervals around the differences between the two compared means (Rothman and Ericson, 1987). We rejected the null hypothesis of equal means if the computed Scheffé intervals did not contain the number zero.

Second, we examined each chemical class separately for potential variation due to differences in structure or functional group, using Repeated Measures ANOVA and tracking sex, compound and sex x compound interactions as factors. These analyses were performed using square root transformed mean EAG data for each compound within chemical classes. For GLVs and aromatic compounds, we performed some follow-up comparisons of mean EAGs for select groups of related compounds using Student's *t*-tests adjusted for multiple comparisons with the sequential Bonferroni method described by Rice (1989), testing null hypotheses of no statistical differences between means. Male and female data were pooled for *t*-tests, because there were no significant ANOVA terms associated with sex (see below). For aromatics, we performed a second series of ANOVA on groups of compounds conforming to the following four classes: benzoic acid methyl esters, *ortho*-hydroxyl-benzoic esters (salicylates), benzaldehydes and phenylpropanoids. Follow-up tests focused on two groups of compounds sharing a common carbon skeleton but differing by functional group or carbon chain length.

We analyzed dose-response EAG data (% of standard) with respect to three specific parameters: threshold dose, maximum EAG response and slope of the dynamic response phase (see Light et al., 1992b). Threshold dose represents the lowest dose at which a stimulant evokes a mean EAG response distinguishable from responses to controls. The dynamic response phase describes the stimulus dose interval during which the greatest change or increase in EAG responsiveness (i.e., slope) is observed.

Antennal Morphological Data

We performed a number of morphological measurements on male and female *H. lineata* moths to determine whether sexual dimorphism in body size is reflected in antennal morphology. We measured dry body mass and forewing length (base to apex; a good surrogate for body size, see Haber and Frankie, 1989) of all experimental moths, and compared these measures using one-tailed Student's *t*-tests, with a null hypothesis of female moths not being significantly larger than males. Then, we measured the mass, length, diameter, and number of segments (annuli) of the left antenna of each moth. Antennal diameter was measured under a dissecting microscope (50X) using a glass slide calibrated to

0.1 mm; all other measurements were made with a ruler calibrated to 1.0 mm. Dry body and antennal masses were measured using a Mettler AE 100 analytical balance calibrated to 0.0001 g. Antennal measurements were adjusted for differences between male and female body size and compared using ANCOVA (Systat 5.2). The ANCOVA model considered antennal mass, diameter, length, and segment number as separate dependent variables, with sex as a treatment and dry body mass and forewing length as covariates.

RESULTS

General Antennal Responsiveness

The mean −mV responses of *H. lineata* male (mean ± SE: −0.18 ± 0.02 mV) and female (−0.24 ± 0.03 mV) antennae to hexane solvent "control" stimulations did not differ significantly ($t = 1.521$, $P = 0.08$), but the responses were generally larger and more variable for females than for males. Similarly, males' (−0.22 ± 0.04 mV) and females' (−0.30 ± 0.07 mV) EAG responses to the 1% linalool standard stimulus did not differ significantly, although neither sex's responses were uniform. All test compounds elicited measurable EAGs that were greater than those for hexane controls in male and female moths.

Responses to Clarkia breweri Floral Volatiles

Each of the 10 *C. breweri* floral volatiles elicited measurable EAG responses, but the largest EAGs in both sexes (females > −0.8 mV; males > −0.45 mV) were observed in response to benzyl acetate, methyl salicylate and linalool (Figure 1A). Female EAGs were larger than those of males for all of the *C. breweri* compounds, nearly two-fold larger in the cases of eugenol, methyl salicylate and pyranoid linalool oxide (Figure 1A). Benzyl benzoate elicited EAGs from both sexes that were similar in magnitude to those for eugenol and methylisoeugenol, but benzyl benzoate should be regarded as the most potent stimulant of the three, due to its large size (MW 212) and low volatility (b.p. 323°C; see Appendix 1). Male and female EAG responses to methylisoeugenol, vanillin, and veratraldehyde were the lowest among this group of compounds, less than or equal in magnitude to those for the standard stimulus.

Responses to Other Floral Volatiles

Mean male and female EAG responses to the 22 additional floral scent compounds ranged in magnitude from very high (e.g., 2-methyl-butyraldoxime, < −0.8 mV; Figure 1B) to barely detectable (e.g., myrcene, ≤ −0.15 mV). Female EAGs were greater than those of males for all compounds except *allo-*

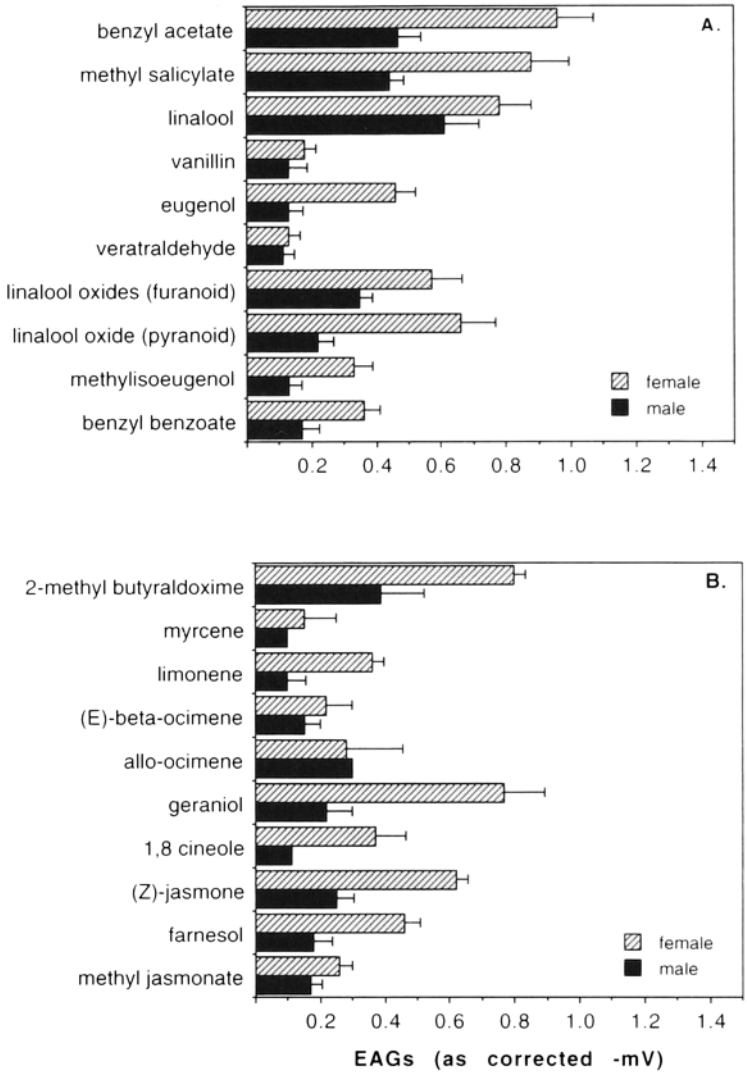


FIG. 1. Female and male *H. lineata* EAG responses to individual scent compounds, expressed as mean corrected $-$mV $\pm$ SE (see text). A. Floral headspace scent compounds from *Clarkia breweri*. B, C. Floral scent compounds from other moth-pollinated plant species; B. Terpenoids and fatty acid derivatives and C. Aromatic or benzenoid compounds. D. "Green Leaf Volatiles."

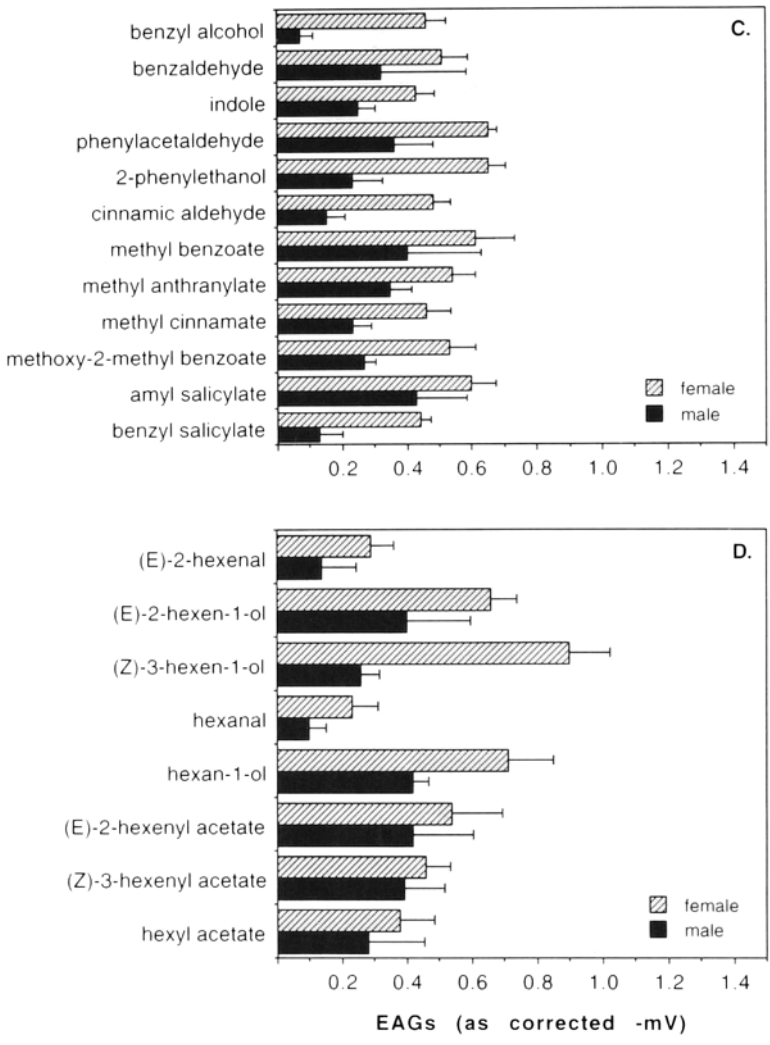


FIG. 1. Continued.

ocimene, especially so in the cases of benzyl alcohol, cinnamic aldehyde, and geraniol (Figures 1B, C). When *H. lineata* EAG magnitudes are scaled to differences in relative volatility, as related to molecular weight and boiling point (Appendix 1), stimulant compounds such as 2-methyl-butyraldoxime (MW 102) and farnesol (MW 222) are comparable in potency, while large compounds (MW

> 200) evoking strong EAG responses, such as amyl salicylate and (*Z*)-jasmone (Figures 1B, C), must be considered among the most highly potent antennal stimulants tested.

Responses to Green Leaf Volatiles (GLVs)

EAG responses to aliphatic alcohols and acetates were moderate to large in magnitude (> -0.4 mV) in both males and females, but EAG responses to the aldehydes hexanal and (E)-2-hexenal were relatively small (Figure 1D). Females' and males' EAG responses to most compounds were similar in magnitude (Figure 1D). The rank order of GLV response potency was: C_6 alcohols $\geq$ C_8 acetates $\geq$ C_6 aldehydes.

Rank Orders of EAG Responses

The rank orders of male and female EAG responsiveness (% of standard) to stimulant compounds at ca. 100 ug dosage are given in Table 1. Male and female ranks were significantly correlated (Spearman's Rank Correlation Coefficient = 0.715, $P < 0.001$), but marked differences in rank were observed for a number of the compounds, particularly within the top ten ranks (see Table 1). Because female EAGs were greater than those of males for most compounds, some differences in rank order were due to high male responsiveness to compounds that were relatively poor female stimulants (e.g., amyl salicylate, *allo*-ocimene, (*E*)-2- and (*Z*)-3-hexenyl acetate, hexyl acetate, linalool; see Table 1). The remaining differences in rank order were attributable to lower male responsiveness to compounds that were strong olfactory stimulants to females (e.g., benzyl alcohol, geraniol, (*Z*)-3-hexen-1-ol, pyranoid linalool oxide, 2-phenylethanol).

Chemical Class Comparisons

When EAG responses to the individual compounds were combined as pooled, averaged and derived indexes for structural chemical classes (Figure 2, Table 2), variation between these classes was significant ($P < 0.001$, Repeated Measures ANOVA). However, the effects of sex and sex x class interactions were not significant (see Table 2A). Monoterpenes elicited the smallest mean EAG magnitudes as a pooled compound class, and these responses were significantly smaller than those elicited by oxygenated terpenoids (Scheffé interval contrast, see Table 2B). However, grand mean EAG responses to aromatic esters were not significantly larger than those to aromatic alcohols and aldehydes, nor were mean responses to aromatic volatiles as a whole statistically separable from those to all grouped terpenoids (Table 2B).

TABLE 1. RANK ORDERS OF *Hyles lineata* EAG RESPONSES TO SCENT COMPOUNDS, EXPRESSED AS % OF STANDARD: FEMALES VS. MALES

Chemical class compound	Female rank	Male rank
Aromatics		
amyl salicylate	14	14
benzaldehyde	18	25
benzyl acetate	2	5
benzyl alcohol	16	39
benzyl benzoate	32	32
benzyl salicylate	33	29
(*E*)-cinnamic aldehyde	20	30
eugenol	27	27
indole	11	21
methoxy-2-methylbenzoate	19	12
methyl anthranylate	15	13
methyl benzoate	21	18
methyl cinnamate	29	17
methylisoeugenol	35	28
methyl salicylate	3	4
phenylacetaldehyde	12	9
2-phenylethanol	7	16
vanillin	38	37
veratraldehyde	39	34
Fatty-acid derivatives		
hexanal	34	33
hexan-1-ol	9	2
(*E*)-2-hexenal	28	36
(*E*)-2-hexen-1-ol	5	7
(*Z*)-3-hexen-1-ol	1	8
(*E*)-2-hexenyl acetate	17	6
(*Z*)-3-hexenyl acetate	24	3
hexyl acetate	22	26
(*Z*)-jasmone	10	23
2-methylbutyraldoxime	4	11
methyl jasmonate	31	22
Terpenoids		
1,8 cineole	23	38
farnesol	26	20
geraniol	6	24
limonene	30	31
linalool	8	1
linalool oxide (Z-pyranoid)	13	15
linalool oxides (*Z/E* furanoid)	25	10
myrcene	40	40
allo-ocimene	37	19
(*E*)-*beta*-ocimene	36	35

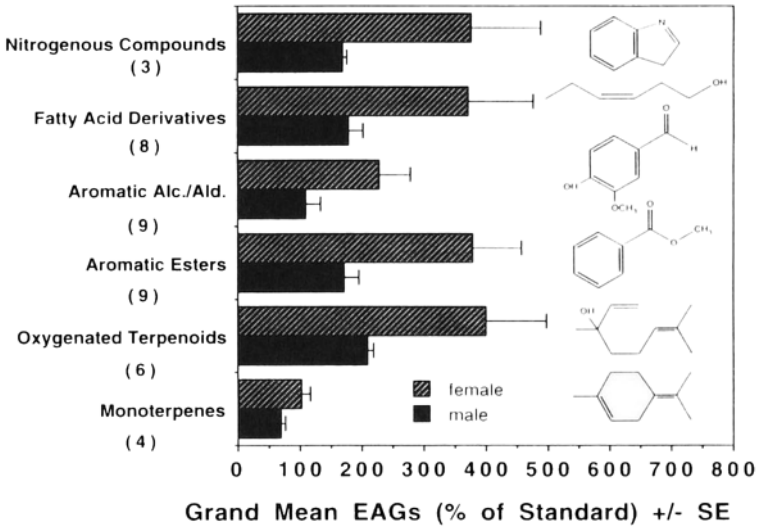


FIG. 2. Summary of *Hyles lineata* moths' EAG responsiveness to different chemical classes, as derived from pooling and averaging the mean EAG responses to each individual test compound belonging to a given chemical class (see Appendix 1). Relative responses are expressed as % of response to standard stimulus (1% v/v linalool in hexane). Number of compounds pooled for each chemical class is given in parentheses. Representative chemical structures are given for each class.

Variation Within Chemical Classes

Significant levels of variation were identified within the aromatic ester, aromatic aldehyde/alcohol, aliphatic GLV, monoterpene, and oxygenated terpene chemical classes (Repeated Measures ANOVA, Table 3). The effects of sex and sex x compound interactions were not found to be significant within most compound classes, with the exception of monoterpenes. This result is most likely due to the small number of compounds in this group (four) and especially strong sex differences in response to limonene (Figure 1B). Among aromatic sub-classes, significant compound-level variance was detected for the salicylates and benzoic acid methyl esters, but not for the benzaldehyde- and phenylpropanoid-related compounds (Table 4).

Follow-up tests of GLV compounds included comparisons of mean EAG responses to compounds varying in the chain position and configuration of C=C double bounds (e.g., (*Z*)-3-hexen-1-ol vs. (*E*)-2-hexen-1-ol) and to C_6 and C_8 GLV compounds sharing the same basic carbon skeleton but differing in degree of saturation, such as hexanal and (*E*)-2-hexenal (Student's *t*-tests, $P \leq 0.005$

TABLE 2. COMPARISON AMONG POOLED, DERIVED, SQUARE-ROOT TRANSFORMED MEAN EAG RESPONSES (% OF STANDARD STIMULUS)

A. Repeated Measures ANOVA

Category	Factor	SS	DF	MS	F	P
Between subjects	sex	227.43	1	227.43	3.07	0.13
	error	444.51	6	74.09		
Within subjects	class	322.44	5	64.49	14.35	<0.001
	class × sex	21.72	5	4.34	0.97	0.45
	error	134.86	30	4.50		

B. *A Priori* Scheffé Contrasts

Comparison	Grand means (transformed)	Scheffé[a] interval
Monoterpenes vs oxyg. terpenoids	9.33 17.54	8.17 ± 3.17
Aromatic esters vs arom. aldehydes and alcohols	16.74 13.03	3.70 ± 3.71
Aromatics vs terpenoids	14.89 13.46	1.43 ± 2.63

[a]Null hypothesis of equal means is rejected if Scheffé interval does not contain the number zero.

for multiple comparisons; Table 5A). None of the five comparisons presented in Table 5A resulted in significantly different mean EAG responses.

Although we observed significant variation in EAG responses to aromatic esters (ANOVA, $P = 0.02$, Table 4), individual differences between methyl benzoate and its *ortho*-substituted derivatives were not statistically significant in pairwise *t*-test comparisons (Table 5B). We did not perform other possible pairwise comparisons within this subclass. We also observed significant variation in EAG responses among salicylates (ANOVA, $P < 0.001$, Table 4). EAG responses to methyl salicylate (with a methyl ester) were significantly greater than those to the larger benzyl salicylate (with a benzyl ester), but not significantly different from those for amyl salicylate (Table 5B).

Dose Responses to Clarkia breweri Volatiles

Dose-response EAG curves for male and female responses to test compounds were similar in shape but varied in their threshold, slope, and maximum -mV response (Table 6). Threshold doses for both sexes were on the order of 10^{-4} $\mu g/\mu l$ for linalool, pyranoid linalool oxide, and methyl salicylate (also

TABLE 3. VARIATION WITHIN COMPOUND CLASSES, REPEATED MEASURES ANOVA[a]

Compound class	Factor	SS	*df*	MS	*F*	*P*
Aromatic esters (N=9)	sex	497.56	1	497.56	3.26	0.12
	error	916.91	6	152.82		
	compound	789.39	8	98.67	7.79	**<0.001**
	cmpd. × sex	100.70	8	12.59	0.99	0.45
	error	607.73	48	12.66		
Aromatic aldehydes & alcohols (N=9)	sex	481.22	1	481.22	3.52	0.11
	error	830.68	6	136.78		
	compound	595.65	8	74.46	5.40	**0.004**
	cmpd. × sex	254.60	8	31.83	2.31	0.08
	error	661.47	48	13.78		
Aliphatic GLV's (N=8)	sex	284.76	1	284.76	1.79	0.23
	error	956.33	6	159.39		
	compound	876.28	7	125.18	7.79	**<0.001**
	cmpd. × sex	174.00	7	24.86	1.55	0.18
	error	675.24	42	16.08		
Nitrogen-bearing compounds (N=3)	sex	185.08	1	185.08	2.15	0.19
	error	516.40	6	86.07		
	compound	49.96	2	24.98	2.98	0.09
	cmpd. × sex	13.14	2	6.57	0.78	0.48
	error	100.56	12	8.38		
Monoterpenes (N=4)	sex	13.21	1	13.21	245.18	**0.004**
	error	0.11	2	0.05		
	compound	140.80	3	46.93	18.89	**0.002**
	cmpd. × sex	45.28	3	15.10	6.08	**0.03**
	error	14.91	6	2.48		
Oxygenated terpenoids (N=6)	sex	238.51	1	238.51	2.11	0.20
	error	677.62	6	112.94		
	compound	312.47	6	62.49	2.96	**0.03**
	cmpd. × sex	72.44	5	14.49	0.69	0.64
	error	634.07	30	21.14		

[a]Abbreviations: SS = sum of squares, *df* = degrees of freedom, MS = mean square. Significant *p* values are given in bold face.

benzyl acetate for females), 10^{-3} for methylisoeugenol, 10^{-2} for benzyl benzoate, and 10^{-1} for eugenol (Figure 3A–C). Males were sensitive to lower threshold doses of furanoid linalool oxides (10^{-2} $\mu g/\mu l$) than were females (threshold = 10^{0} $\mu g/\mu l$), while females had lower thresholds (10^{-4} $\mu g/\mu l$) to benzyl acetate than did males ($>10^{-3}$ $\mu g/\mu l$). The highest EAG response max-

TABLE 4. VARIATION WITHIN AROMATIC SUB-CLASSES, REPEATED MEASURES ANOVA[a]

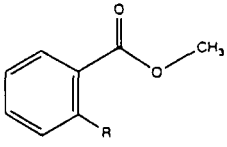
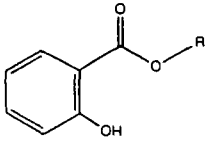
Compound class	Factor	SS	*df*	MS	*F*	*P*
Salicylates (N=3)	sex	253.84	1	253.84	4.97	0.07
	error	306.20	6	51.03		
	compound	411.33	2	205.67	18.14	**<0.00**
	cmpd. × sex	7.30	2	3.68	0.33	0.73
	error	136.09	12	11.34		
Benzoic acid esters (N=4)	sex	160.73	1	160.73	1.83	0.23
	error	527.72	6	87.95		
	compound	248.98	3	83.99	5.46	**0.02**
	cmpd. × sex	17.35	3	5.78	0.38	0.69
	error	273.86	18	15.22		
Benzal-dehydes (N=3)	sex	46.41	1	46.41	1.78	0.23
	error	156.45	6	26.07		
	compound	159.49	2	79.74	4.35	0.06
	cmpd. × sex	78.78	2	39.39	2.15	0.18
	error	220.15	12	18.35		
Phenyl-propanoids (N=4)	sex	143.90	1	143.90	2.56	0.16
	error	336.71	6	56.12		
	compound	53.45	3	17.82	2.42	0.11
	cmpd. × sex	53.17	3	17.73	2.41	0.11
	error	132.30	18	7.35		

[a]Abbreviations: SS = sum of squares, *df* = degrees of freedom, MS = mean square. Significant *p* values are given in bold face.

ima for both sexes were observed for benzyl acetate, linalool, and methyl salicylate. These compounds evoked the steepest slopes of EAG response curves over the broadest dynamic response ranges (10^{-1} to 10^{2} $\mu g/\mu l$ concentrations; Table 6).

Male and female EAG rank orders for eight *C. breweri* compounds at doses from 10^{2} down to 10^{-4} $\mu g/\mu l$ are listed in Table 7. Mean rank orders over all doses were comparable to those listed for doses of 10% (Table 1), with some inconsistencies resulting from different threshold doses among stimulants (e.g., furanoid linalool oxides). Compounds such as linalool and methyl salicylate were consistently strong antennal stimulants throughout the range of doses tested. Benzyl acetate was a relatively poor stimulant at dosages below 10^{-2} $\mu g/\mu l$, while pyranoid linalool oxide was a relatively strong antennal stimulant at doses below 10^{0} $\mu g/\mu l$.

TABLE 5. COMPARISONS OF ISOMERS AND FUNCTIONAL GROUPS WITHIN CHEMICAL CLASSES[a]

Comparison	Student's t	P
A. GREEN LEAF VOLATILES		
$C_6H_{12}O$		
(*Z*)-3-hexen-ol vs	−0.49	0.64
(*E*)-2-hexen-1-ol		
$C_8H_{14}O$		
(*Z*)-3-hexenyl acetate vs	0.09	0.93
(*E*)-2-hexenyl acetate		
C_6		
hexan-1-ol vs	0.23	0.82
(*E*)-2-hexen-1-ol		
hexanal vs	−1.20	0.25
(*E*)-2-hexenal		
C_8		
hexyl acetate vs	−1.02	0.32
(*E*)-2-hexenyl acetate		
B. AROMATIC ESTERS		
1. *ortho*-substituted methyl esters of Benzoic Acid		
		
methyl benzoate (R = H) vs	−0.37	0.72
methyl anthranylate (R = NH_2)		
methyl benzoate vs	−2.63	0.02
methyl salicylate (R = OH)		
methyl benzoate vs	−0.26	0.80
methoxy-2-methyl benzoate (R = OCH_3)		
2. Salicylates.		
		
methyl salicylate (R = CH_3) vs	−2.13	0.05
amyl salicylate (R = $CH_2CH_2CH\ CH_3$)		
CH_3		
methyl salicylate vs		
benzyl salicylate (R = $-CH_2-$)	−3.40	**0.004**

[a]Comparisons are two-tailed Student's t-tests comparing square-root transformed mean EAGs (% of standard). Experiment-wide significance level is $P \leq 0.005$, after Bonferroni adjustment for multiple comparisons. Significant p-values given in bold print.

TABLE 6. DOSE-RESPONSE PARAMETERS, *Hyles lineata* EAG SENSITIVITY (% OF STANDARD) TO *Clarkia breweri* FLORAL VOLATILES (A = FEMALE, B = MALE)

Stimulatory compound	% of *C. breweri* floral scent[a]		Log threshold dosage[b]	Max. response	Dynamic response phase	
					slope	interval
linalool	27.63	A.	< -4	428 %	109	10^{-1}–10^{2}
		B.	< -4	448 %	133	10^{-1}–10^{2}
linalool	0.77	A.	0	160	80	10^{0}–10^{2}
oxides (furanoid)		B.	< -2	180	52	10^{-1}–10^{2}
linalool	10.72	A.	< -4	117	73	10^{0}–10^{1}
oxide (pyranoid)		B.	> -4	125	117	10^{0}–10^{1}
benzyl acetate	42.15	A.	-4	718	214	10^{-1}–10^{2}
		B.	> -3	296	123	10^{0}–10^{2}
benzyl benzoate	3.93	A.	-2	109	45	10^{0}–10^{2}
		B.	> -2	75	25	10^{1}–10^{2}
methyl	3.32	A.	< -4	598	166	10^{-1}–10^{2}
salicylate		B.	< -4	375	117	10^{-1}–10^{2}
eugenol	1.32	A.	> -1	109	55	10^{-1}–10^{1}
		B.	> -1	100	51	10^{-1}–10^{1}
methylisoeugenol	1.44	A.	< -3	92	35	10^{-1}–10^{1}
		B.	< -3	62	21	10^{-1}–10^{1}

[a]Percent of floral headspace composition, 24 hr scent collection over Tenax TA/charcoal, GC-MS analysis: Raguso and Pichersky, 1995. Vanillin and veratraldehyde were not used in dose-response experiments.
[b]Dosage of compound in hexane solution ($\mu g/\mu l$) as applied to filter papers in odor delivery cartridges.

Morphological Measurements

Female *H. lineata* moths were significantly larger than males, both in terms of dry body mass ($P = 0.007$) and forewing length ($P = 0.002$; one-tailed *t*-test, Table 8A). However, this inequality in body size was not reflected in antennal morphology. After scaling for body size differences using ANCOVA, female and male antennal diameter, antennal mass, and antennal segment number were not significantly different, but male antennae were significantly longer than those of females, relative to body size ($P = 0.014$, Table 8b).

DISCUSSION

Sexual Dimorphism and Rank Order

Antennae of adult male and female *H. lineata* responded in a sensitive and dynamic manner to the presentation of chemically diverse olfactory stimulants.

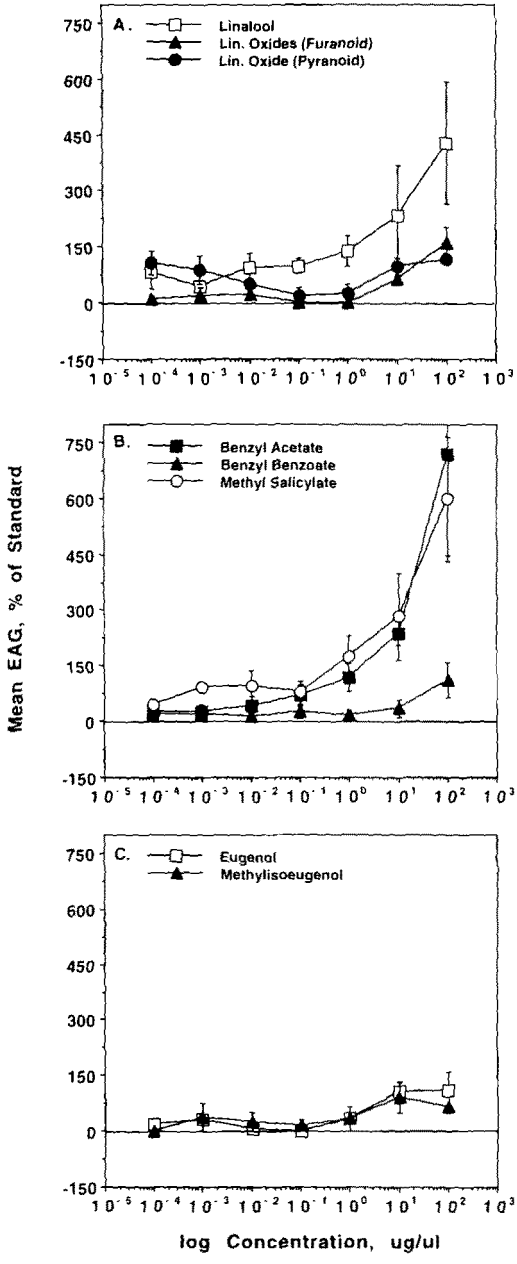


FIG. 3. Dose-response curves of female (A–C) and male (D–F) *H. lineata*, showing EAG responses to *C. breweri* floral volatiles at different v/v concentrations (as applied to filter paper wicks), expressed as mean % of standard ± SE vs log μg/μl.

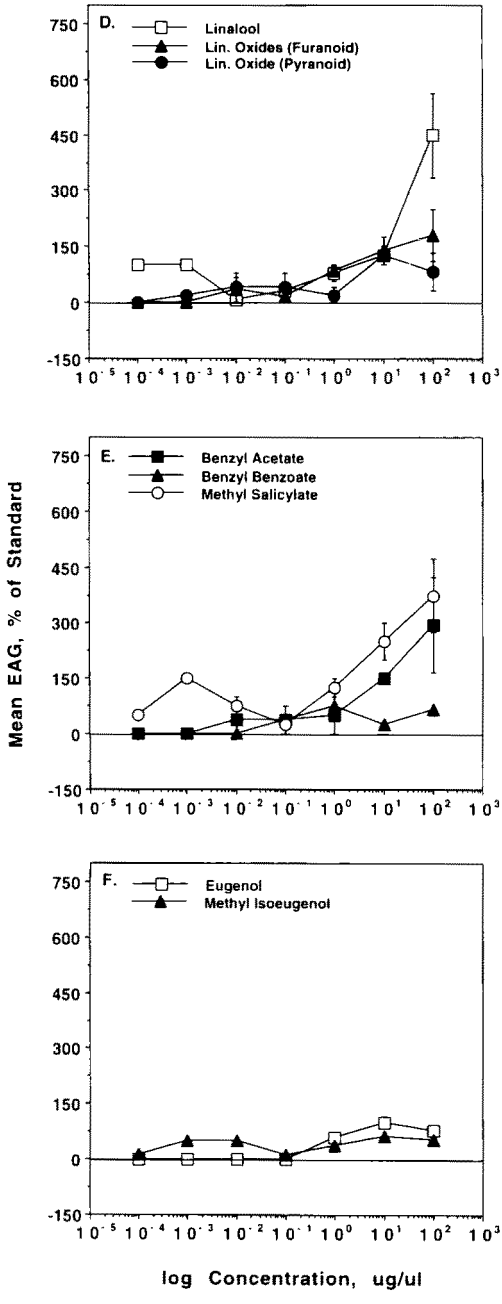


FIG. 3. Continued.

TABLE 7. RANK ORDERS OF EAG RESPONSES (% OF STANDARD) TO DIFFERENT CONCENTRATIONS OF *Clarkia breweri* FLORAL SCENT COMPOUNDS[a]

Stimulatory compound	Log concentration ($\mu g/\mu l$) per filter paper														$\bar{X}$	
	10^{2}		10^{1}		10^{0}		10^{-1}		10^{-2}		10^{-3}		10^{-4}			
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
benzyl acetate	3	1	2	2	6	3	2	3	4	4	-	6	-	4	3	3
benzyl benzoate	7	6	8	8	4	7	3	4	-	6	-	-	-	-	6	7
eugenol	6	7	6	4	5	5	8	-	-	-	-	-	-	-	8	5
linalool	1	3	4	3	3	2	4	1	6	2	2	3	1	2	2	2
linalool oxides (furanoid)	4	4	3	7	2	8	6	-	5	-	-	-	-	-	4	8
linalool oxide (pyranoid)	5	5	5	5	8	6	1	5	3	3	4	2	-	1	5	4
methylisoeugenol	8	8	7	6	7	4	7	6	2	5	3	4	-	-	7	6
methyl salicylate	2	2	1	1	1	1	5	2	1	1	1	1	2	3	1	1

Note: Dashes indicate that compound could not be ranked at that dosage because it was below the threshold of detection.

[a] M = male (N = 3), F = female (N = 6).

Female EAGs were greater than those of males for most compounds, sometimes dramatically so (Figures 1A–D), yet sex was not a significant factor in most Repeated Measures ANOVA between and within chemical classes (Tables 2–4). The female-biased disparity in EAG responses to our experimental volatiles was not a scaling artifact due to the overall larger body size of females, because female antennae were actually smaller than those of males relative to body size (Table 8). However, variation within sexes was large in general (see error bars, Figures 1A-D), probably due to the sample sizes and differences in the moths' ages and experiences in the wild; thus, sex was not a significant factor in most of our ANOVA tests. Male and female EAG rank orders were significantly correlated (Table 1), and the shapes, thresholds, and slopes of the dose/response curves for the *Clarkia breweri* floral volatiles were fundamentally the same for both sexes, with responses of females being slightly larger in magnitude (Figures 3A–F, Tables 6, 7). These results suggest that male and female antennae are endowed with homologous types of olfactory receptors tuned to a wide range of "floral" and "vegetative" odorants. The few EAG rank order discrepancies observed among males and females (e.g., higher male responses to *allo*-ocimene; higher female responses to geraniol) may reflect (1) specific sex-related quantitative differences in antennal receptor neuron populations, (2) qualitative differences in olfactory physiology, or (3) altered EAG receptivities resulting from adult moth experiences prior to capture in the wild

TABLE 8. COMPARISON OF MALE AND FEMALE *H. lineata* BODY SIZE AND ANTENNAL MORPHOLOGY[a]

A. Sexual dimorphism in body size (one-tailed Student's *t* test)

Category (units)	Mean measurement (± SE)			
	Males	Females	*t*	*P*
Dry body mass (g)	0.250 (± 0.019)	0.340 (± 0.021)	3.14	**0.007**
Forewing length (mm)	32.3 (± 0.9)	37.4 (± 1.0)	3.76	**0.002**

B. Comparison of antennal morphological measurements using ANCOVA

Treatment	SS	*df*	MS	*F*	*P*	Adjusted Measures	
						sex	LSM ± SE
1. Antennal diameter (mm)							
Sex	0.007	1	0.007	2.226	0.170	female	0.75 ± 0.03
Dry mass	0.000	1	0.000	0.083	0.780	male	0.82 ± 0.03
Forewing	0.000	1	0.000	0.015	0.905		
Error	0.028	9	0.003				
2. Segment number							
Sex	10.378	1	10.378	0.307	0.593	female	60.72 ± 2.79
Dry mass	13.788	1	13.788	0.408	0.539	male	58.00 ± 3.11
Forewing	1.490	1	1.490	0.044	0.808		
Error	304.256	9	33.806				
3. Antennal dry mass (g)							
Sex	0.005	1	0.005	2.896	0.123	female	0.0013 ± 0.0002
Dry mass	0.002	1	0.002	1.094	0.323	male	0.0018 ± 0.0002
Forewing	0.000	1	0.000	0.139	0.718		
Error	0.015	9	0.002				
4. Antennal length (mm)							
Sex	3.031	1	3.031	9.326	**0.014**	female	12.24 ± 0.27
Dry mass	1.475	1	1.475	4.538	0.062	male	13.72 ± 0.30
Forewing	0.383	1	0.383	1.179	0.306		
Error	2.925	9	0.325				

Note: Numbers in bold face are statistically significant at $P \leq 0.05$. Sample sizes: females, N = 7; males, N = 6.

[a] Abbreviations: ANCOVA = analysis of covariance, SS = sum of squares, *df* = degrees of freedom, MS = mean square, LSM = least squares mean, SE = standard error.

(see Vet et al., 1990; De Jong and Pham-Delegue, 1991). We have no data bearing on this question at present.

Previous observations of sex-bias in pheromone response (Schweitzer et al., 1976; Reed et al., 1987; Christensen et al., 1989), floral visitation (Kislev et al., 1972; Knudsen and Tollsten, 1993) and host plant orientation and ovi-

position (Yamamoto et al., 1969; Tichenor and Seigler, 1980) in *Manduca sexta* (L.) and other hawkmoth species suggest that complex sexual dimorphism might exist for scent compound receptors in the antennae of nectar-feeding hawkmoths. For example, while both sexes of *M. sexta* visit flowers, females respond to solanaceous host plant volatiles during oviposition behavior (Tichenor and Seigler, 1980), while males respond to female sex pheromone (Schweitzer et al., 1976; Hildebrand et al., 1992), detected by the specific *sensilla trichoidea* receptors that physically and numerically dominate their antennae (up to 69% of all receptors per antennal segment; Lee and Strausfeld, 1990). Thus, greater female EAG magnitudes in *H. lineata* might reflect either larger populations of antennal receptors tuned to plant volatiles (see Mayer et al. 1984), or greater chemosensory efficiency in females, either due to the reduced size of trichoid *sensilla* on their antennae (Lee and Strausfeld, 1990) or to differences in aerodynamic flow of scent-laden air over the differently shaped antennae (M. Willis, pers. comm.). Kislev et al. (1972) suggest that male-bias in floral visitation by *H. lineata* in Israel is due to differences in male and female responses to certain floral scent compounds. Our results, however, support Knudsen and Tollsten's (1993) contention that this bias is not explainable by innate differences in peripheral olfactory reception of floral volatiles. If anything, female *H. lineata* antennae appear to be slightly more receptive to most floral scent compounds than are those of males.

EAG Responses and Chemical Classes

We observed strong EAG responses to 10% volumetric concentrations of a wide variety of scent compounds emitted by moth-pollinated flowers, including aromatic esters, aldehydes, and alcohols, oxygenated monoterpenoids, a sesquiterpenol, fatty-acid derivatives and assorted aliphatic and aromatic nitrogen-bearing compounds. Similarly, Brantjes (1973) recorded large EAGs using two other hawkmoth species (*Deilephila elpenor* L. and *M. sexta*), in response to 13 individual floral compounds (mostly terpenoids and aromatics) and to floral scent extracts from nine plant species, but his data were not presented quantitatively. Collectively, these results demonstrate that nectar-feeding hawkmoths are capable of detecting a wide range of floral and vegetative volatiles from diverse chemical classes. This broad olfactory vocabulary includes floral compounds (e.g., 2-phenylethanol and phenylacetaldehyde) that typify noctuid moth-pollinated flowers such as *Silene vulgaris* (Caryophyllaceae; Pettersson, 1991; Knudsen and Tollsten, 1993) and *Abelia grandiflora* (Caprifoliaceae; Haynes et al., 1991). Indeed, these and other "noctuid" flowers are often visited by sphingids (Nilsson, 1983; Pettersson, 1991; Wasserthal 1993).

We observed strong EAG responses to eight aliphatic GLV compounds, particularly to C_6 alcohols and C_8 acetates. Many of these compounds also are

potent EAG stimulants for *M. sexta* (Schweitzer et al., 1976; Light unpubl. data) and *Pieris* butterflies (van Loon et al., 1992). However, the GLVs are smaller and more volatile molecules than most of the aromatic and terpenoid test compounds; thus, the number of GLV molecules delivered during a stimulatory puff would be proportionally higher than that of larger, less volatile odorants. Although vegetative/leaf volatiles are clearly important to ovipositional decision-making in female *M. sexta* (Yamamoto et al., 1969; Ramaswamy, 1988), the role of GLVs as potential olfactory stimulants during *Hyles* oviposition bouts has not yet been examined. *H. lineata* is extremely polyphagous throughout its range, utilizing plants from the Nyctaginaceae, Onagraceae, Polygonaceae, Portulacaceae, Rosaceae, Scrophulariacaea, and Vitaceae as ovipositional sites and larval hosts (Wiltshire, 1957; Hodges, 1971; Common, 1990). Host species or genus-specific vegetative volatiles appear to function as important olfactory cues to females of *M. sexta*, which oviposit only on Solanaceae (Tichenor and Seigler, 1980), but host choice by gravid *Hyles* females may depend more on general cues, such as the ubiquitous GLVs and terpenes such as caryophyllene, myrcene, and pinene (Visser et al., 1979; Light et al., 1992a, 1992b, 1993; van Loon et al., 1992).

Functional Group Variation

Significant differences in antennal responsiveness correlated with structural variation in pheromone or plant volatile chemistry have been observed in hawkmoths (Reed et al., 1987), butterflies (van Loon et al., 1992) and other phytophagous insects (see Light et al., 1988, 1992a, 1992b). In our experiments, the importance of specific functional groups in determining EAG response potency depended strongly on the particular carbon skeleton geometry and/or chemical class. For example, oxygenated monoterpenoids elicited significantly higher EAG responses than did monoterpenes (Figures 1A, B, Table 2), the chief difference among them being the presence of a hydroxyl group (e.g., linalool vs. myrcene). Among the aliphatic GLVs, our results document a clear superiority in EAG potency of aliphatic alcohols over aldehydes. However, differences in levels of carbon skeleton saturation and double bond (C=C) geometry were not associated with statistically significant EAG differences.

We detected significant amounts of variation in EAG responses to diverse aromatic esters, aldehydes, and alcohols (Table 3), especially to benzoic acid esters and salicylates (Table 4), yet few specific functional group comparisons resulted in significantly different EAG responses. Benzaldehyde was a more potent antennal stimulant than either of the substituted benzaldehydes, vanillin and veratraldehyde, especially for female moths (Figs. 1A, C), but these differences did not translate into significant within-class variance (ANOVA, Table 4). Similarly, we did not detect significant variance associated with differences

in *para*-position oxidation among such compounds as eugenol, methylisoeugenol, vanillin, and veratraldehyde (ANOVA, Table 4).

Pairs of aromatic compounds that varied in the presence or absence of *ortho*-substituted hydroxyl-groups, such as methyl benzoate vs. methyl salicylate and benzyl benzoate vs. benzyl salicylate did not differ significantly. Methyl anthranylate, methyl salicylate and methyl-2-methoxybenzoate share a common skeleton but substitute amino-, hydroxyl- and methoxyl- groups, respectively, for the *ortho*-position hydrogen of methyl benzoate. However, mean EAG responses to these compounds did not differ significantly from those to methyl benzoate itself. Among salicylates, methyl salicylate elicited significantly higher EAGs than did benzyl salicylate, but this result could be an artifact of benzyl salicylate's higher molecular weight (40% larger) and lower volatility (Appendix 1). From these data, we are unable to infer whether *H. lineata* moths possess separate antennal receptors for chemically similar plant volatiles such as methyl salicylate and methyl anthranylate, which are quite distinct to the human palate (oil of wintergreen and concord grape, respectively), or whether functional group differences bear any physiological or behavioral importance to these insects.

Dose-Response Sensitivities

Foraging hawkmoths undoubtedly encounter a rich and broad spectrum of plant odors while in flight and during floral visitation. The dose-response data suggest that *H. lineata* moths are sensitive to most *C. breweri* floral compounds at a dose of ca. 1 ng on filter paper. This keen receptivity supports the possibility that scent compounds function as long-distance attractants to foraging hawkmoths (Tinbergen, 1958; Bratjes, 1973). Our results mirror those of Van Loon et al. (1992; *Pieris* butterflies) in showing that EAG rank orders may change with different stimulant dosages (e.g., eugenol and methylisoeugenol; Figure 3C, F, Table 6), suggesting that certain plant volatiles may have different behavioral or physiological activities at different concentrations. In the absence of behavioral bioassays or choice experiments, however, it is difficult to determine physiologically relevant odorant concentrations as emitted from a flower. The identification of floral volatiles using dynamic headspace sorption is usually used as a qualitative analytical procedure (Bergström et al., 1980; Bicchi and Joulain, 1990), but at best provides quantitative data only on cumulative floral odor emission per unit fresh weight over time (usually 12–24 hours; Raguso and Pichersky, 1995). These studies provide little if any inference (and usually an overestimation) of the actual concentrations of floral scent compounds encountered by foraging moths in a wind-borne odor plume (Murlis et al., 1992). Subtle changes in scent production or concentration, similar to changes in color, may convey information on nectar reward or physiological condition to a foraging insect (Weiss, 1991; Dobson, 1994). Thus, antennal sensitivity to floral scent

compounds across a range of concentrations might be as important to a moth during a floral visit as it is to a moth flying upwind in search of a flower.

EAGs and the C. breweri-H. lineata Interaction

In our study of the *C. breweri-H. lineata* pollination interaction, we have identified aromatic esters (benzyl acetate and methyl salicylate) and oxygenated monoterpenoids (linalool, linalool oxides) as the principal floral volatiles (>80% of GC-MS peak area; Raguso and Pichersky, 1995) and the most potent antennal chemostimulants. Aromatic esters and oxygenated terpenoids are among the most common scent components identified from hawkmoth-pollinated flowers spanning diverse plant families (Loughrin et al., 1990; Kaiser, 1991, 1993; Knudsen and Tollsten, 1993). Aromatic esters, in particular, are suspected to function as floral attractants of many hawkmoth species (Morgan and Lyon, 1928; Hodges, 1971; Nilsson, 1983), but they are not restricted to the aromas of night-blooming flowers (Knutsen and Tollsten, 1993). The presence of the uncommon N-bearing oxime compounds and oxygenated sesquiterpenes (e.g., farnesol and nerolidol) in many temperate and tropical hawkmoth-pollinated flowers has led many authors to suggest that these compounds function as hawkmoth attractants (Nilsson et al., 1985; Kaiser, 1993; Knudsen and Tollsten, 1993). Our data do not suggest anything unusual about these compounds at the level of antennal detection (see Figures 1B, C).

The EAG is believed to represent the sum of receptor potentials elicited in all sensory neurons by stimuli presented to the antennae (Boeckh et al., 1965; Schweitzer et al., 1976; Mayer et al. 1984). In essence, a discernible EAG response greater than the control stimulation indicates that a moth's antenna is sensitive to that odorant at the trial dosage. However, the behavioral activities of compounds can only be identified through controlled behavioral bioassays conducted in field or laboratory settings (Dodson et al., 1969; Jacobson et al., 1976; Tichenor and Seigler, 1980; Haynes et al., 1991).

CONCLUSIONS

Our EAG studies of *Hyles lineata* olfaction firmly establish that these moths can detect all of the floral scent compounds of *Clarkia breweri* at physiologically relevant concentrations. These results are consistent with the hypothesis that hawkmoths can detect the floral scent of this plant from a distance. In addition, male and female moths demonstrated a broad olfactory vocabulary, showing strong EAG responses to a variety of aromatic, aliphatic, terpenoid and nitrogen-bearing scent compounds found in the floral and vegetative tissues of many plants utilized by hawkmoths as nectar and oviposition resources. Considering

these findings, the behavioral responses of hawkmoths to diverse floral odors are not likely to result from gross differences in antennal receptor physiology, but rather from differences in individual experiences and/or central nervous system organization. The hypothesis that the floral scent of *C. breweri* could function as a long-distance attractant cannot be rejected by these findings, but EAG data are not sufficient to demonstrate behavioral attraction, arrestment, or repulsion, but only the capacity for olfactory detection. Behavioral experiments using live hawkmoths in wind tunnels or flight cages will be required to test this hypothesis.

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APPENDIX 1. TEST COMPOUNDS, PURITIES, SOURCES, AND JUSTIFICATION FOR INCLUSION IN STUDY

Compound (Class[1])	Formula	MW	B.P. °C	Purity	Source	Justification[2]
Aromatic Compounds						
benzyl alcohol (1)	C_6H_6O	94	205	92%	Eastman/White	3,4
benzaldehyde (1)	C_7H_6O	106	178	99%	Aldrich	3,4
indole (6)	C_8H_7N	107	254	95%	Eastman	3
phenylacetaldehyde (1)	C_8H_8O	120	194	–	Aldrich	4
2-phenylethanol (1)	$C_8H_{10}O$	122	205	–	Eastman/White	4
(*E*)-cinnamic aldehyde (1)	C_9H_8O	132	248	95%	R. Flath	3,5
methyl benzoate (2)	$C_8H_8O_2$	136	200	98%	Fritzche	3
benzyl acetate (2)	$C_9H_{10}O_2$	150	214	99+%	Aldrich	1,3,4
methyl (2,6) anthranylate	$C_8H_9O_2N$	151	*	–	CPL	5
methyl salicylate (2)	$C_8H_8O_3$	152	224	98%	Aldrich	1,3
vanillin (1)	$C_8H_8O_3$	152	285	99%	Aldrich	1
methyl cinnamate (2)	$C_{10}H_{10}O_2$	162	262	99%	Aldrich	5
eugenol (1)	$C_{10}H_{12}O_2$	164	255	99%	Aldrich	1

APPENDIX 1. Continued

Compound (Class[a])	Formula	MW	B.P. °C	Purity	Source	Justification
veratraldehyde (1)	$C_9H_{10}O_3$	166	281	99%	Aldrich	1
methoxy-2-methyl benzoate (2)	$C_9H_{11}O_3$	167	245	-	R. Flath	3,5
methyl-isoeugenol (1)		178	*	99%	Aldrich	1
amyl salicylate (2)	$C_{12}H_{16}O_3$	208	*	75%	TCI	3,5
benzyl benzoate (2)	$C_{14}H_{12}O_3$	212	323	-	Sigma	1,3
benzyl salicylate (2)	$C_{14}H_{12}O_3$	228	*	99%	ICN	3,5
Fatty-Acid Derivatives						
(*E*)-2-hexenal (3)	$C_6H_{10}O$	98	*	99%	Aldrich	2
(*E*)-2-hexen-1-ol (3)	$C_6H_{12}O$	100	159	97%	Aldrich	2
(*Z*)-3-hexen-1-ol (3)	$C_6H_{12}O$	100	157	98%	Aldrich	2
hexanal (3)	$C_6H_{12}O$	100	131	99%	Aldrich	2
hexan-1-ol (3)	$C_6H_{14}O$	102	157	98%	Aldrich	2
2-methyl-butyraldoxime (3,6)	$C_5H_{12}ON$	102	*	-	R. Flath	3,4
(*E*)-2-hexenyl acetate (3)	$C_8H_{14}O_2$	142	*	98%	Aldrich	2
(*Z*)-3-hexenyl acetate (3)	$C_8H_{14}O_2$	142	*	98%	Aldrich	2
hexyl acetate (3)	$C_8H_{16}O_2$	144	169	98%	Aldrich	2
(*Z*)-jasmone (3)	$C_{11}H_{16}O$	164	258	70%	ICN	3
methyl jasmonate (3)	$C_{13}H_{20}O_3$	224	*	-	R. Flath	5
Terpenoids						
limonene (4)	$C_{10}H_{16}$	136	176	99%	Aldrich	3
myrcene (4)	$C_{10}H_{16}$	136	167	98%	Aldrich	3,5
(*E*)-*beta*-ocimene (4)	$C_{10}H_{16}$	136	178	97%	R. Flath	3
allo-ocimene (4)	$C_{10}H_{16}$	136	*	86%	Fluka	5
linalool (5)	$C_{10}H_{18}O$	154	198	97%	Sigma	1,3
1,8 cineole (5)	$C_{10}H_{18}O$	154	177	99%	Aldrich	3
geraniol (5)	$C_{10}H_{18}O$	154	230	98%	Aldrich	1,3
linalool oxides (*E*/*Z*-furanoid) (5)	$C_{10}H_{18}O_2$	170	*	99%	Aldrich	1,3
linalool oxide (*Z*-pyranoid) (5)	$C_{10}H_{18}O_2$	170	*	99%	KLS	1,3
farnesol (*E*/*Z* mix) (5)	$C_{15}H_{26}O$	222	*	95%	Aldrich	3

[a]Chemical classes assigned as follows:
1 = aromatic alcohols and aldehydes
2 = aromatic esters
3 = fatty-acid derived compounds
4 = monoterpenes
5 = oxygenated terpenoids
6 = N-bearing compounds (aromatic and aliphatic)

APPENDIX 1. Continued

[a] Justification for inclusion in study:
1. present in floral headspace GC-MS analysis of *Clarkia breweri* (Raguso and Pichersky, 1995).
2. "green leaf volatiles", ubiquitous in vegetation.
3. present in many hawmoth-pollinated, night-blooming flowers (Knutsen and Tollsten, 1993).
4. present in many noctuid moth-pollinated flowers (Haynes et al., 1991; Knutsen and Tollsten, 1993).
5. similar to other test compounds but varying in one functional group (see text).

[b] Boiling point data at atmospheric pressure not available in Merck Index, CRC Handbook of Chemistry and Physics, Sigma/Aldrich Handbook of Chemical Safety.

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EXOGENOUS JASMONATES SIMULATE INSECT WOUNDING IN TOMATO PLANTS (*Lycopersicon esculentum*) IN THE LABORATORY AND FIELD

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Abstract—Wounding increases the levels and activities of several defense-related proteins in the foliage of the tomato plant, *Lycopersicon esculentum* Mill. Evidence indicates that two of these responses, the systemic increases in polyphenol oxidase and proteinase inhibitors, are regulated by an octadecanoid-based signalling pathway which includes the wound hormone, jasmonic acid. It is not known whether other responses to wounding are also regulated by this same signalling pathway. In this paper, we show that application of jasmonates (jasmonic acid or its volatile derivative, methyl jasmonate) in low concentrations to foliage of young tomato plants induced, in a dose-dependent manner, the same protein responses–polyphenol oxidase, proteinase inhibitors, lipoxygenase, and peroxidase–as does *Helicoverpa zea* Boddie feeding. Application of jasmonic acid to a single leaflet of four-leaf tomato plants induced these four proteins in a spatial pattern nearly identical to that produced by localized feeding of *H. zea*. Exogenous jasmonic acid also decreased suitability of foliage for the beet armyworm, *Spodoptera exigua* Hubner in the laboratory. Based on these results, we conducted an experiment to measure the effects of jasmonic acid spray under field conditions. We provide the first evidence that jasmonic acid spray on field plants induces production of chemical defenses above the levels found in unsprayed controls. Exogenous jasmonic acid sprayed on plants in agricultural plots increased levels of polyphenol oxidase and proteinase inhibitors. Because application of jasmonic acid induces these defensive compounds at low concentrations in a manner similar to natural wounding, it may prove to be a useful tool for stimulating plant resistance to insects in the field.

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Key Words—Jasmonic acid, methyl jasmonate, induced phytochemistry, induced resistance, polyphenol oxidase, peroxidase, lipoxygenase, proteinase inhibitors, *Helicoverpa zea*, *Spodoptera exigua*.

INTRODUCTION

In many plants, localized wounding increases allocation to defensive chemistry in wounded tissues as well as in distant, unwounded portions of the plant (Baldwin, 1994). Systemic responses of this sort require a mobile signal(s), generated at the site of wounding, to regulate their expression. Several putative signal molecules have been identified, among them the linolenic acid derivative, jasmonic acid (JA) (Reinbothe et al., 1994). In many plant species, wounding increases endogenous levels of JA, and exogenous JA stimulates the expression of defensive chemistry (Reinbothe et al., 1994). The latter fact suggests that it may be possible to use applications of JA to stimulate induced plant resistance on a large scale in the field without damaging individual plants.

In foliage of the tomato, *Lycopersicon esculentum* Mill, caterpillar feeding initiates an array of defense-related responses which exhibit a characteristic spatial pattern (Stout et al., 1996). The most prominent systemic responses are the increases in the levels and activities of polyphenol oxidase (PPO) and of two proteinase inhibitors (PIs). Local responses include increased activity of the oxidative enzyme lipoxygenase (LOX) in addition to the PPO and PI responses. Peroxidase (POD), another oxidative enzyme, is also induced, but less markedly and consistently, by caterpillar feeding. All of these proteins are thought to decrease the nutritive value of tomato foliage to herbivores, particularly noctuid larvae (Duffey and Felton, 1991). Evidence indicates that the most prominent protein responses–the systemic increases in the levels of PIs and PPO–are coregulated at the transcriptional level by an octadecanoid-based signal transduction pathway which includes JA (Farmer et al., 1992; Constabel et al., 1995). Application of JA or its volatile derivative, jasmionic acid methyl ester (methyl jasmonate, MJ) to tomato foliage causes increased production of PI and PPO mRNA and proteins (Farmer et al., 1992), and wounding increases endogenous JA levels in young tomato plants (Doares et al., 1995). It is unknown, however, whether other chemical responses to caterpillar feeding are controlled by the same signalling pathway as the PPO/PI response, or whether these other responses are controlled by separate signalling pathways. It is also not known whether the increase in plant resistance to subsequent herbivores which occurs as a result of caterpillar feeding and which is presumably causally related to one or more of the chemical changes can be simulated by application of JA.

In this paper, we show that application of jasmonates to tomato foliage induces lipoxygenase and peroxidase activities in addition to proteinase inhibitors and polyphenol oxidase, and show that the spatial pattern of this chemical

induction is nearly identical to the spatial pattern of induction by feeding of the noctuid, *Helicoverpa zea*. We also demonstrate that exogenous JA causes a systemic increase in plant resistance to herbivory which is similar to that caused by *H. zea* feeding. These results prompted us to test the effects of exogenous JA on the defensive chemistry of field-grown tomato plants. We report here that application of JA to tomato plants in large field plots induced both polyphenol oxidase and proteinase inhibitors. Our results show that jasmonic acid may be a useful tool for increasing plant resistance on a large scale in agricultural conditions.

MATERIALS AND METHODS

Laboratory Experiments

Plant Growth. Tomato plants (*Lycopersicon esculentum* CV. "Castlemart") were used at the four-leaf stage in all experiments (23–27 days after planting). Plants were grown in a greenhouse in soil, watered daily, and fertilized weekly with a 15-30-15 P-N-K fertilizer. Greenhouse temperature was regulated at 32°C ± 5°; natural light was not supplemented.

Chemicals. Jasmonic acid methyl ester was purchased from Bedoukian Research, Inc. (Danbury, CT) and, when necessary, converted to jasmonic acid following the method of Farmer et al. (1992). All other chemicals were from Sigma.

Application of Jasmonates to Plants. Plants were treated with MJ by saturating cotton-tipped applicators in an ethanol:MJ (9:1) solution and placing the applicators in a growth chamber without physically contacting the plants to be treated. Control plants were placed in a separate growth chamber. Plants were kept in the growth chambers for 12 hours, after which they were placed in a greenhouse until chemical assays were performed. Both growth chambers were maintained at 25°C and plants were maintained under light during exposure to MJ.

For JA experiments, JA was dissolved in circa 1 ml/gm of acetone, then dispersed in water to the desired concentration. JA was sprayed on plants until run-off using an atomizer; if necessary, surrounding leaflets were shielded with a piece of paper. Control plants were sprayed with water which contained acetone at the same concentration used for the JA treatment.

Effect of Exogenous Jasmonates on Plant Chemistry in the Laboratory. The effects of JA and MJ on the activities of tomato defensive chemistry were evaluated in a series of experiments conducted in the spring of 1995.

Initial experiments were conducted to determine the effect of exposure to MJ on the activities of the putative defenses PPO, POD, LOX, and PIs. Plants

assigned to the MJ treatment were exposed to an MJ-rich atmosphere for 12 hours as outlined above, removed to a greenhouse for an additonal 36 hours, then assayed for protein activities. Leaflets from the third leaf of both control and treated plants were used for protein assays. This experiment was performed twice, with 11 plants/treatment, and the growth chambers used for the two treatments were switched in the two experiments.

Local induction of exogenous JA (i.e., induction in leaves which had had contact with JA) was assessed by spraying the third leaves of four-leaf plants with solutions of JA of several concentrations (0 mM, 0.1 mM, 1 mM, and 10 mM). Leaves were sprayed in a greenhouse and the plants kept in the greenhouse for 48 hours before performing protein assays. Protein activities were assayed only from leaves which had had contact with JA. Leaves from eight plants were sprayed per concentration.

Systemic induction by JA was compared to systemic induction by feeding of larval *H. zea* (corn earworm) in three experiments. For the first two experiments, terminal leaflets of third leaves were sprayed with JA (0.5 mM) or wounded with larval *H. zea* by confining a fourth-instar larva to the leaflet with a clip cage. A third group of plants was sprayed with water containing acetone as a control. For the third experiment, the insect feeding treatment was omitted. Two days after spraying/wounding, leaflets from four positions on the plant were harvested for chemical analyses: the wounded/treated leaflet, a leaflet adjacent to the treated leaflet, a leaflet from the leaf below the treated leaflet, and a leaflet from the leaf above the treated leaflet.

Effect of Induction on Herbivore Performance in the Laboratory. Two short-term (one day) and one longer-term (seven days) feeding assays were used to test the effect of induction by jasmonates on the suitability of foliage for the noctuid *Spodoptera exigua* Hubner. *S. exigua* larvae were obtained as eggs from the USDA (Stoneville, MS) and raised on artificial diet until the late second to early fourth instar, depending on the assay (approximately 6–10 days). To initiate asays, larvae were taken off diet, starved for two to six hours (to allow guts of larvae to void), weighed, and chosen for uniformity of size. Starved larvae were placed with one or two leaflets from either treated or untreated plants on moist filter paper in a petri dish and allowed to feed. To terminate the assay, larvae were removed from leaflets and weighed after starving them for one hour. One short-term experiment utilized leaflets from the third leaves of control or MJ-treated plants; the second short-term experiment used unsprayed leaflets from JA-sprayed or control third leaves. In the third experiment, leaflets were from JA-sprayed or unsprayed third leaves and insects were allowed to feed for seven days. Leaflets were switched every two or three days and leaflets for the JA treatment were taken from plants sprayed two or three days earlier. Relative growth rates were calculated using fresh weights according to the methods of Waldbauer (1968).

Field Experiments

Effect of Exogenous Jasmonic Acid on Plant Chemistry in the Field. A one-quarter acre field of tomato plants (*L. esculentum* CV "Ace") containing sprayed and unsprayed plots was established in May 1995. The field was randomly divided into 2 × 3 m plots; 30 replicates of each treatment. The field was maintained by the Department of Vegetable Crops, University of California, Davis, CA. The tomatoes were planted in ten beds spaced one meter apart and 70 meters long. The beds were pretreated with herbicide, fertilized, manually weeded, and furrow watered weekly for the duration of the experiment. When the seedlings emerged they were thinned so that they were six inches apart and as the plants grew they continued to be thinned so that mature plants were spaced every two feet. Our field was surrounded by tomato fields on two sides, a road and unplanted fields on the others.

The treatment plots were first sprayed with JA on June 1, when the plants were in the five leaf stage, and subsequently on June 13, June 28, and July 25, until the chemical effects of induction were no longer detactable. On June 1, 13, and July 25, JA was sprayed at 0.5 mM, and on June 28 at 0.25 mM. With each application, circa 0.125–0.250 micromoles of JA per plant was applied using a backpack sprayer. Control plants were sprayed with a water/acetone solution as in the laboratory experiments.

Chemical assays were conducted throughout the summer. Assays were conducted two–three days following each spray and at irregular intervals between sprays. In young plants, the third terminal leaflet was collected. In larger plants, old (fourth oldest) and young leaflets were collected. Young leaves were marked with string before fully expanded and used for assays the following week to standardize age. Leaflets were excised from plants using a razor and transported on ice to the laboratory for chemical analysis. Fifteen plants were selected from each treatment and for all assays, plants were assayed only once.

An additional plot of tomatoes was established in September 1995 to determine if young plants were inducible late in the season. Tomato seedlings were grown in the greenhouse for one month and then transplanted outside into a 100 ft long row surrounded by open space. Plants were spaced every 8 inches and each plant was randomly assigned to the spray or no spray treatment. A pre-treatment assay was conducted to ensure that PPO levels were uniform between plants before the treatments were imposed (N = 12 per treatment). PPO was assayed two weeks following spray (N = 15 per treatment).

Chemical Assays

Protein Assays. Enzymes were extracted by homogenizing weighed leaflets in 1.25 ml of ice-cold 0.1 M K Phos buffer containing 7% (w:v) polyvinyl-polypyrrolidine, then adding 0.4 ml to 10% (v:v) Triton X-100 and vortexing

the mixture. The homogenate was centrifuged at 10,000 *g* for ten min and the resulting supernatant used directly for spectrophotometric assays of PPO, POD, and LOX activities. For PPO assays, 20–30 μL of enzyme extract was added to 1 ml of a caffeic acid solution (2.92 mM in pH 8 K Phos buffer) and the increase in OD at 470 nm of the mixture measured. POD activity was also measured as an increase in OD_{470} after addition of 20–30 μL of enzyme extract to 1 ml of a substrate solution consisting of 2.92 mM guaiacol in pH 8 K Phos buffer with H_2O_2 added as a cofactor. The LOX assay measured the appearance of conjugated dienes at 234 nm (Hildebrand and Hymowitz, 1981). The reaction mixture consisted of 15–20 μL of enzyme extract added to 2.9 ml of 0.4 mM linoleic acid dispersed in a 0.1 M K Phos buffer (pH 7) containing Tween-20 (0.1%). Change in absorbance was monitored for at least 5 min. The activities of all three enzymes are expressed as ΔOD/min/gm of fresh leaf tissue.

The PI assay was based on the ability of plant extracts containing proteinase inhibitors to inhibit the digestion of the artificial protease substrate benzoyl tyrosine ethyl ester (BTEE) by commercial proteases. Proteinase inhibitors were extracted from individual leaflets by grinding them in a 50 mM Tris HCl buffer (pH 7.8, 3 ml/gm leaf tissue) containing 7% polyvinylpolypyrrolidine, 1.67 mM phenylthiourea, 0.3 M KCl, and 0.4 mM ascorbic acid. This extract was immediately frozen for later use. For assays, the thawed extract was centrifuged at 13,000 *g* for ten min and the supernatant used as the source of PIs. In a quartz cuvette, 25 μL of the PI extract was added to 25 μL of 0.001 N HCl containing 0.001 mg of chymotrypsin and the mixture allowed to incubate for ten min. After incubation, 2.9 ml of 0.5 mM BTEE was added to the cuvette and the increase in absorbance at 256 nm measured. For every run of samples, a control consisting of chymotrypsin and BTEE without plant extract was also run, and PI activities of samples expressed as % chymotrypsin activity relative to this control. The relationship between % inhibition of chymotrypsin and concentration of proteinase inhibitor is linear for a wide range of proteinase inhibitor concentrations (Stout, unpublished data).

RESULTS

Effects of MJ on Tomato Foliar Chemistry

Short-term exposure to MJ vapors had marked effects on the activities of defense-related proteins in tomato foliage. The effects of MJ on PPO and PI activities were particularly pronounced, with approximately three-fold induction of both protein activities in both trials (Table 1). Exposure to MJ caused a two-fold induction in POD activity in the second trial but not in the first (Table 1). LOX activity was also higher, but not significantly so, in MJ-treated plants than in control plants.

TABLE 1. EFFECTS OF EXPOSURE TO METHYL JASMONATE VAPORS ON THE ACTIVITIES OF FOUR PUTATIVE DEFENSIVE PROTEINS IN TOMATO FOLIAGE. ACTIVITIES WERE FROM THIRD LEAVES OF FOUR-LEAF PLANTS. RESULTS FROM TWO EXPERIMENTS ARE SHOWN. MEANS IN THE SAME COLUMN FOLLOWED BY DIFFERENT LETTERS ARE STATISTICALLY SIGNIFICANT ($p < 0.05$ BY ANOVA)

	Protein activities (± SE)[a]			
Treatment	PPO	POD	LOX	PIs
1/13/95				
Control	12.49 ± 1.28 a	6.72 ± 0.97 a	1.38 ± 0.14 a	25.23 ± 2.73 a
MJ-treated	34.74 ± 2.25 b	7.74 ± 1.54 a	1.81 ± 0.33 a	69.43 ± 9.61 b
2/2/95				
Control	9.16 ± 0.87 a	4.62 ± 0.89 a	1.12 ± 0.21 a	12.50 ± 2.97 a
MJ-treated	43.90 ± 3.76 b	8.14 ± 1.220 b	2.10 ± 0.56 a	71.23 ± 4.86 b

[a]Enzyme activities are expressed as ΔOD/min/gm, PI activities as % inhibition of chymotrypsin activity relative to a control run with chymotrypsin and substrate only (see methods)

Local Induction by Exogenous JA in the Laboratory

Leaves which had been sprayed with JA did not differ in appearance from unsprayed leaves except at the highest concentration of JA (10 mM), which caused leaf necrosis and was clearly phytotoxic. Contact with JA at the lower concentrations increased the activities of PPO and PIs in a dose-dependent manner, but there was no further increase in the activities of these proteins at the highest concentration (Figure 1). LOX activities also increased with increasing JA concentrations, with a sharp rise at the highest concentration (Figure 1). POD activities, in contrast, were not affected by contact with JA except at the highest concentration (Figure 1).

Systemic Induction by Exogenous JA in the Laboratory

Because the battery of responses induced by jasmonate–PPO, PIs, LOX, and, inconsistently, POD–was the same as that resulting locally from caterpillar feeding (Stout et al., 1996), we next asked whether the pattern of systemic induction resulting from a localized application of JA was the same as the systemic pattern of induction resulting from localized caterpillar feeding. To answer this question, we compared the local and systemic induction caused by spraying 0.5 mM JA on the terminal leaflet of the third leaf with the induction caused by *H. zea* feeding at the same leaflet position. Protein activities were compared in the damaged leaf (enzymes only), in undamaged leaflets from the damaged leaf, in leaflets from a leaf below the damaged leaf, and in leaflets

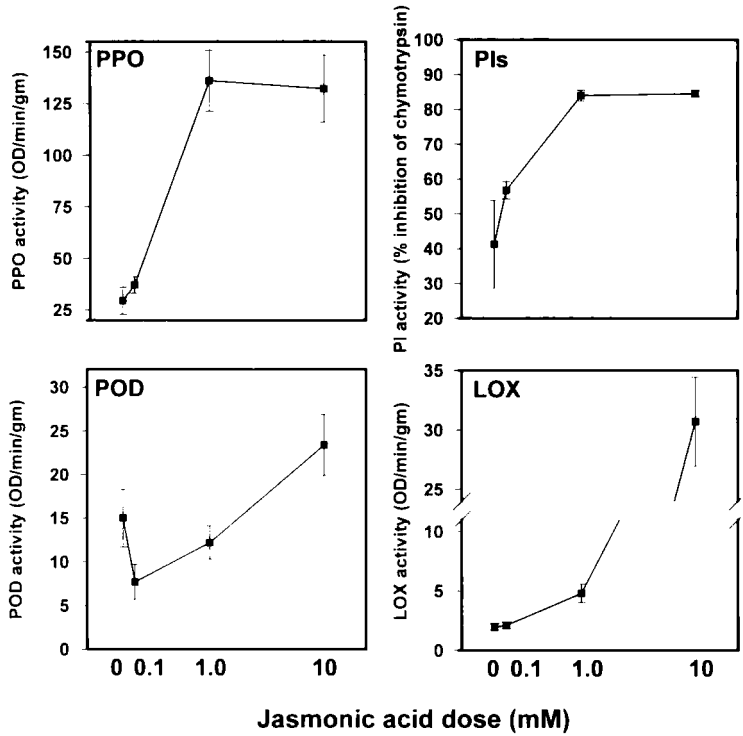

FIG. 1. Effect of several concentrations of jasmonic acid on the activities of the proteins polyphenol oxidase, proteinase inhibitors, lipoxygenase, and peroxidase. Jasmonic acid was suspended in water and applied to leaflets as a spray; each point represents the mean activity from eight sprayed leaflets each from a different plant. Means accompanied by different letters are significantly different ($p < 0.05$; one-way ANOVA followed by a Tukey mean separation).

from a leaf above the damaged leaf. In general, the pattern of responses to JA mirrored the responses to *H. zea* feeding (Table 2). Induction of PPO–in terms of the ratio of PPO activity in control plants to PPO activity in damaged/treated plant–was strongest in the damaged leaf and undamaged leaflets of the damaged leaf (approximately two- to twelve-fold), present but weaker in the leaf below the damaged leaf (1.5- to nine-fold), and weakest or absent in the leaf above the damaged/treated leaf (at most, three-fold). Induction of PIs was more uniformly systemic in both JA-treated and damaged plants. Induction of LOX, in contrast, was confined to the damaged leaflet except in one instance of *H. zea* feeding. POD was induced inconsistently by both JA and caterpillar feeding,

TABLE 2. EFFECT OF LOCALIZED APPLICATION OF JASMONIC ACID (0.5 mM) AND LOCALIZED *H. zea* FEEDING ON PROTEIN ACTIVITIES ± SE AT FOUR POSITIONS ON FOUR LEAF TOMATO PLANTS. REPRESENTATIVE RESULTS FROM THREE EXPERIMENTS ARE SHOWN. MEANS IN THE SAME COLUMN FOLLOWED BY DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT ($p < 0.05$; ANOVA FOLLOWED BY A TUKEY MEAN SEPARATION)

Treatment	Protein activities[a] ± SE at leaflet position[b]			
	Damaged/treated	Adjacent	Lower	Upper
Polyphenol oxidase				
Control	6.99 ± 0.65 a	9.29 ± 1.26 a	2.27 ± 0.87 a	17.94 ± 5.76 a
Jasmonic acid	55.50 ± 4.01 b	62.14 ± 5.55 b	17.88 ± 4.09 b	43.84 ± 6.12 b
H. zea	39.84 ± 5.48 b	41.66 ± 5.14 b	5.35 ± 1.34 a	19.82 ± 3.82 a
Proteinase inhibitors				
Control	n/a	42.3 ± 14.2 a	58.7 ± 4.9 a	38.9 ± 8.5 a
Jasmonic acid	n/a	86.4 ± 7.8 b	78.0 ± 8.9 b	89.9 ± 4.8 b
H. zea	n/a	86.3 ± 2.4 b	92.5 ± 8.4 c	88.5 ± 7.2 b
Lipoxygenase				
Control	0.48 ± 0.08 a	0.92 ± 0.11 a	0.43 ± 0.05 a	0.75 ± 0.10 a
Jasmonic acid	2.09 ± 0.24 b	0.95 ± 0.10 a	0.47 ± 0.05 a	1.10 ± 0.17 a
H. zea	2.03 ± 0.36 b	1.14 ± 0.07 a	0.58 ± 0.10 a	2.37 ± 0.27 b
Peroxidase				
Control	14.80 ± 1.44 a	12.53 ± 0.98 a	17.81 ± 1.29 a	14.84 ± 1.43 a
Jasmonic acid	16.80 ± 1.09 a	15.10 ± 2.14 a	21.06 ± 2.02 a	15.41 ± 0.62 a
H. zea	29.52 ± 5.58 b	19.60 ± 2.94 a	25.92 ± 3.77 a	19.01 ± 2.65 a

[a] Enzyme activities are expressed as ΔOD/min/gm fresh leaf tissue; PI activities are expressed as %inhibition of chymotrypsin activity relative to a control run with chymotrypsin and BTEE only (see methods).

[b] Four-leaf plants were wounded or treated at the terminal leaflet of the third leaf. Activities were assayed from the damaged/treated leaflet, a leaflet adjacent to the treated leaflet, a leaflet from a leaf below the treated leaflet, and a leaflet from the leaf above the treated leaflet. There was insufficient leaf material available to assay both enzyme and PI activities at the damaged position.

and induction by both treatments was strongest in the damaged leaf. POD induction was weaker than that of PPO, LOX, and PIs.

Effect of Jasmonate Exposure on Plant Resistance

Larvae reared on leaflets from MJ- and JA-treated plants in the laboratory exhibited lower relative growth rates (gm/gm/day) than larvae reared on control leaflets of the same age in all three feeding experiments (Figure 2). The reduction in growth rate on jasmonate-treated plants ranged from 25–80%. This between-treatment difference was seen despite variation in growth rates between experiments, which presumably occurred because of differences in the age of larvae

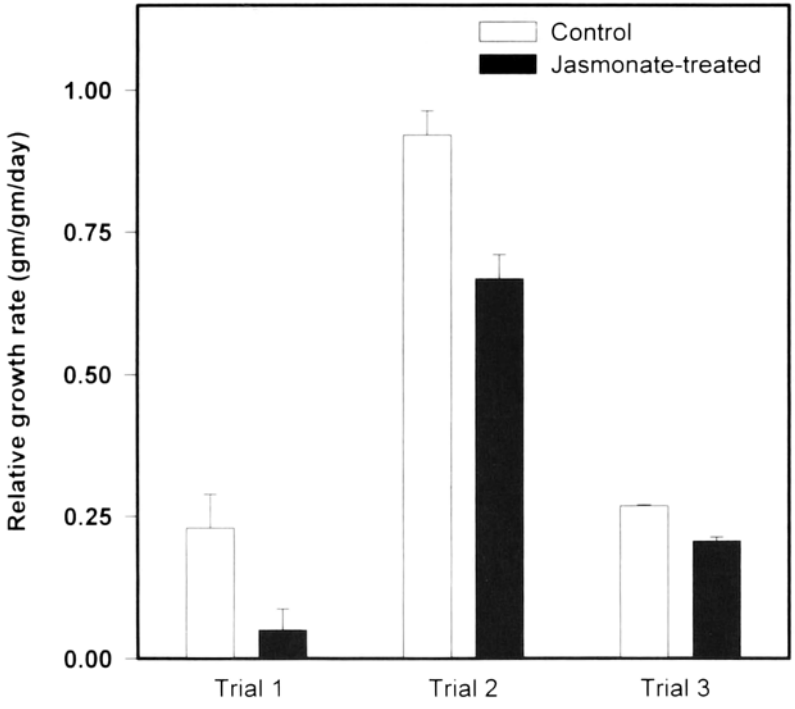

FIG. 2. Relative growth rates ± SE (gm/gm/day) of *Spodoptera exigua* larvae raised on excised control and jasmonate-treated leaflets. In all cases, third leaves of four-leaf plants were used. In the first experiment, plants were treated by exposing them to methyl jasmonate vapors, while spray applications of jasmonic acid were used in the other experiments. The first two assays are short-term (one day) assays; the last experiment lasted seven days. The differences in growth rates between treatments are significant for all three experiments. The number of replicates per treatment ranged from 11 to 16 in each experiment.

used and because of differences in the conditions and methods of the assays. This reduction in relative growth rates of insects on leaflets from sprayed plants was probably due in part to a reduction in consumption rate, which was lower in both of the JA feeding assays (data not shown).

Systemic Induction by Exogenous Jasmonic Acid in the Field

Jasmonic acid spray increased levels of PPO in old and young leaves of young plants (Figure 3A,B). The effect of JA diminished as the plants aged. In early June, differences in PPO activity in sprayed plants were detected in both

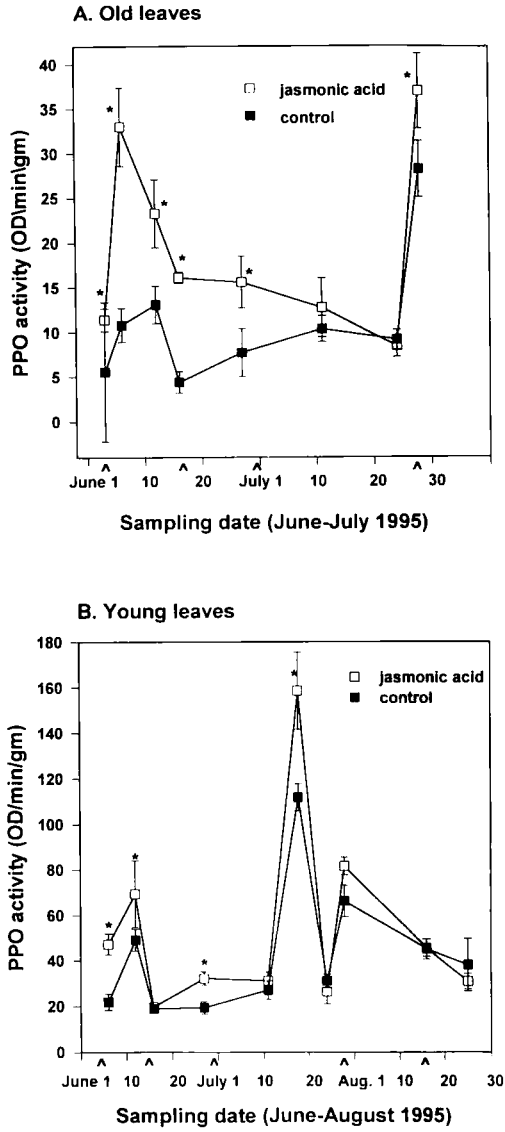

FIG. 3. Polyphenol oxidase levels in old leaves (3A) and young leaves (3B) of field grown plants in summer 1995. Activity in control and jasmonic acid treated plants were compared using a one-way ANOVA on each sample date with 15 replicates of each treatment. Stars indicate significance below the 0.05 level. Arrows along *x*-axis indicate spray dates.

TABLE 3. PROTEINASE INHIBITOR ACTIVITY ± S.E. (% CHYMOTRYPSIN ACTIVITY) IN CONTROL AND JASMONATE TREATED PLANTS GROWN IN THE FIELD. IN ALL CASES, THE TERMINAL LEAFLET OF THE FOURTH LEAF WAS USED. P VALUES WERE CALCULATED USING A ONE-WAY ANOVA

Date	Control	Jasmonic Acid	*F*	P
June 3	44.8 ± 4.1	12.6 ± 0.9	40.2	< .001
June 6	56.6 ± 3.2	22.6 ± 6.1	27.5	< .001
June 16	28.9 ± 7.9	19.5 ± 4.2	1.2	0.28

old and young leaves with young leaves containing higher chemical levels than old leaves. PI activities were higher in sprayed plants on the June 3 and 6 sample dates, but not on June 16 (Table 3). After the June 28 spray, PPO activity was increased in old leaves of sprayed plants, but this effect was not significant. After spraying JA on July 25, PPO activity in old leaves was greater on sprayed plants; young leaves exhibited the same trend but the difference was not statistically significant. In August, PPO activity in young leaves of large plants was not increased by respraying.

JA spray on seedlings planted in September induced higher levels of PPO activity than unsprayed controls ($n = 12$ per treatment, jasmonic acid = 44.57 ± 5.89 (mean ± se), control = 22.43 ± 6.34; df = 1, 22; $F = 6.59$; $p = 0.02$) indicating that the loss of chemical induction in the older plants in August was caused by age and not by season. Chemical differences were not found between the treatments in the pretreatment assay (data not shown).

DISCUSSION

Two approaches have been taken to the study of herbivore-induced plant responses. The first is a mechanistic approach which seeks to determine the chemical and physiological changes induced by herbivory. The second is an ecological approach which examines the impact of induced responses on herbivores. For the most part, these two approaches have not been coupled in a single system. This study represents the first in a series of experiments aimed at determining the effects of artificial stimulation of plant defensive chemistry on populations of herbivores in the field. We show here that jasmonic acid, a putative component of the wound-response pathway in plants, is a valuable tool for manipulating levels of plant defensive chemistry in the laboratory and field. The use of jasmonic acid in the tomato system presents an opportunity to link mechanistic and ecological approaches to induced plant responses.

Application of jasmonic acid to greenhouse grown plants simulated insect wounding in several important respects. The array of chemicals induced by exogenous jasmonic acid was the same as that induced by *H. zea* feeding. Moreover, the spatial distribution of chemical changes following localized application of JA was the same as the spatial distribution of changes following localized *H. zea* feeding. We also found that the chemical changes induced by JA were correlated with a decrease in the suitability of JA-treated plants to *S. exigua* larvae, as occurs following *H. zea* feeding. This increase in foliar resistance was a systemic phenomenon: leaflets used in the two JA feeding experiments had had no contact with JA.

Based on these greenhouse results, we conducted experiments to determine whether artificial application of jasmonic acid in the field induced PPO and PIs, the two most prominent inducible chemicals in greenhouse experiments. Workers have speculated that because plants in the field are subject to many stresses, including insect wounding and physical abrasion, plants in the field may always be induced (Karban and Myers, 1989); however, our data suggest that some plant secondary chemicals remain inducible in the field for two months in spite of these stresses. During the early part of the experiment, both PPO and PIs were strongly induced by exogenous JA (Table 3, Figure 3). PPO remained consistently strongly inducible throughout the first month of the experiment and sporadically inducible during the next month (Figure 3). The loss of PI induction on June 16 (Table 3) may have been because the effect of the jasmonic acid spray wore off or because the plants were induced by other factors.

Many workers have proposed the use of induced resistance as a means of arthropod pest control in agriculture (Kogan and Paxton, 1983; Karban and Baldwin, 1997), but our experiments are the first to demonstrate that plant defenses against insects can be artificially stimulated on a large scale. The artificial stimulation of plant resistance to pathogens using exogenous chemical elicitors is an emerging strategy for pathogen management (Reinbothe et al., 1994; Karban and Baldwin, 1977). Several groups studying the salicylic acid pathway have conducted experiments examining the methods to induce resistance against pathogens in the field (Kessman et al., 1994).

Our greenhouse studies also provide some insights into signalling pathways in tomato. Farmer and Ryan (1992) have forwarded a model for the regulation of wound responses in tomato leaves which involves a lipid-based signalling-system. According to this model, wounding or interaction of plasma membrane receptors with systemic signals (e.g., systemin) causes the release of linolenic acid from the plasma membrane into the cytoplasm. The linolenic acid is then converted by a series of enzymatic reactions to jasmonic acid, which stimulates the expression of defense-related genes. This model was originally generated from data on the proteinase inhibitor response to wounding in tomato foliage; more recent data (Constabel et al., 1995) indicates that the systemic increase in

polyphenol oxidase which occurs following wounding in tomato is also regulated at the transcriptional level by the octadecanoid signal transduction pathway. Our data provide additional support for the hypothesis that JA mediates the PPO and PI responses to wounding, as plants which had received localized applications of JA exhibited higher activities of PPO and PIs in a spatial pattern nearly identical to that produced by localized wounding (Table 2).

Furthermore, our data provide some support for the idea that the lipoxygenase response to caterpillar feeding is also mediated by the octadecanoid pathway. Lipoxygenase activities were consistently induced to higher levels by both JA application and caterpillar feeding; for both treatments, the induction of LOX, unlike the induction of PPO and PIs, was not systemic but was confined to the damaged or treated leaflet. Farmer et al. (1992) presented electrophoretic data which indicated that treating young tomato plants with MJ induced only two proteins in addition to proteinase inhibitors: a 63 kD protein and an 87 kD protein. The former protein is almost certainly polyphenol oxidase (Constabel et al., 1995), while the latter is of similar weight to lipoxygenases from tomato (Zamora et al., 1987). LOX is known to respond to JA treatment in soybean (Creelman and Mullet, 1995).

Although POD activities can be induced by both exogenous jasmonate and *H. zea* feeding, the frequent absence of POD induction in the presence of PPO/PI/LOX induction (Table 2) indicates that the POD response to wounding/JA is probably not regulated in a manner analogous to the PPO/PI responses. Support for this hypothesis comes from the electrophoretic data of Farmer et al. (1992), which showed no proteins of a size consistent with sizes previously reported for peroxidases from tomato and other plants (Marangoni et al., 1989; Bronner et al., 1991). The variability in POD induction, coupled with the fact that strong induction of POD in the dose-response experiment was only observed at the highest concentration of JA, suggests that POD induction may be a secondary result of tissue damage, perhaps involving a separate signalling pathway, rather than a defensive (octadecanoid-mediated) response per se.

The experiments presented in this paper show that jasmonic acid spray in the field simulated the chemical changes caused by natural insect wounding in the laboratory. In future experiments, we will test whether chemical induction by jasmonic acid produces induced plant resistance to herbivores in the field as it does in the laboratory. Preliminary field data indicate that the abundance of aphids and flea beetles is reduced and growth rate of *S. exigua* is slowed on sprayed plants suggesting that artificial chemical induction does increase plant resistance (J. Thaler, unpubl. data).

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HIGH RECAPTURES AND LONG SAMPLING RANGE OF PHEROMONE TRAPS FOR FALL WEB WORM MOTH *Hyphantria cunea* (LEPIDOPTERA: ARCTIIDAE) MALES

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Abstract—Newly emerged males of *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae), marked with different fluorescent colors, were recaptured by pheromone trapping. Three Uni-traps, baited with (3Z,6Z)9,10-epoxyheneicosadiene, (9Z,12Z,15Z)octadecatrienal, and (3Z,6Z)9,10-epoxyheneicosa-triene in a 1:10:1 ratio, total 6 mg/dispenser, were placed in a line 15 m apart, perpendicular to the dominant wind direction, and checked after 12, 36, and 60 hr. At dusk, releases of 10–25 males were made over distances from 30 to 250 m, during 4 periods over a soybean field (nonhost plant). Recapture rates were high; of a total of 176 males released, 115 were recaptured with 88% of these within 12 hours. Between 40 and 100% of males were recovered over shorter distances (30–150 m), and between 10–24% at longer distances (200 and 250 m). No other species or unmarked males were captured. The trapping period showed little effect on recaptures. The central trap of the three traps had somewhat higher catches, but the slope of the regression of recapture over distance did not differ among traps. There was a significant decline of recapture over distance ($r = -0.56$) for catches of individual traps but a large

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scatter. Summed recaptures per distance gave less scatter and had $r = -0.86$, allowing calculation of the maximum sampling range (R_s) after 60 hr as 340 m with a 95% confidence interval of 190–710 m (regression of arcsin $\sqrt{p}$ and $\sqrt{R}$). The area of sampling, found by integrating the probability function of recapture over distance, from the source to maximum R_s was 7 ha. The high recapture rates and the long R_s, compared to those in the literature for other taxa, indicate that pheromone traps are highly efficient sampling devices in this species.

Key Words—Fall web worm moth, *Hyphantria cunea*, Lepidoptera, Arctiidae, pheromone trap, mark-recapture, sampling range.

INTRODUCTION

The range of action of synthetic pheromone traps is one of the most important variables for design of monitoring and mass-trapping schemes (Schlyter, 1992). Traps baited with synthetic sex-pheromone or attractant are today important tools for monitoring of Lepidoptera and other pest insects (Zhang, 1993). Monitoring population dynamics with pheromone traps is usually simpler than other routine measures (Wall, 1989; Wall, 1990; Weslien et al., 1989). However, for any ecological sampling method the area sampled must be defined (Southwood, 1978). The following terms have been used to describe the range of action of an attractant: (1) "active space" (Bossert and Wilson, 1963), (2) "range of sampling" R_S (Wall and Perry, 1987), (3) "range of attraction" R_A (Wall and Perry, 1987), (4) "range of stimulation" (Wall and Perry, 1987), and (5) "effective attraction radius" R_{EA} (Byers et al., 1989). The theoretical "active space" concept is firmly established in the literature, but requires parameters not generally available (Nakamura and Kawasaki, 1977). Wall and Perry (1987) defined the R_S as "the distance from which insects can be shown to reach the source in a given time period," which can be estimated by mark-recapture tests. R_A is "the maximum distance over which insects can be shown to direct their movement to the source" (Wall and Perry, 1987), and requires either direct observations of insects in flight or mark-recapture over short time intervals. The range of stimulation is defined as "the distance over which an attractant source can be shown to elicit a response like wing-fanning" (Wall and Perry, 1987). A simpler concept, the effective attraction radius, R_{EA}, does not take into account any specific behavior of the insect but is defined as the relative trapping power of baited vs. unbaited traps, expressed in terms of the "effective" trap size (radius) of the baited, active trap (Byers et al., 1989). R_{EA} was initially developed for open sticky traps which always give a nonzero catch even on passive traps. In terms of absolute distance (in one dimension) it follows from the definitions that $R_S >$ range of stimulation $\cong$ active space $> R_A > R_{EA}$, at least if the time of study is not very short (Schlyter, 1992).

The fall web worm moth *Hyphantria cunea* (Drury) is one of the most

important pest insects of fruit, nut, and shade trees in China with status as a quarantine pest due to its destructiveness to the host trees. It originated from North America, but was introduced to Eastern Europe and Northeast Asia in this century, and has caused severe damages in its new geographical and host ranges. In China, it has been recorded feeding on over 100 species of deciduous trees (Zhang and Yu, 1985). Only the "black-headed" race has been recognized in Asia (Zhang and Yu, 1985), although two races, a "black-headed" race and a "red-headed" (or "orange") race have been described from North America (Oliver, 1964; Hidaka, 1972). It has two generations per year in China (Zhang and Yu, 1985). A third generation was recorded in 1994, mainly due to extremely high temperatures during the whole summer (Zhang, unpubl.). Male moths engage in "mating flight" in the early morning 0.5–1.0 hr before sunrise in the second generation (Hidaka, 1972; Calcote and Gentry, 1973; Hirooka, 1983; Zhang et al., 1997), but in the early evening in the first generation (Zhang et al., 1997). The identification and synthesis of its sex-pheromone (Hill et al., 1982; Tóth et al., 1989; Mori and Takeuchi, 1989) has permitted various field studies on the biology and applications of pheromone for this moth (Binda et al., 1990; Senda et al., 1993 and references therein; Zhang et al., 1996; Zhang et al., 1997).

For the fall web worm moth Hirooka and Suwanai (1976) predicted with a diffusion equation that the "effective sphere of pheromone" from a virgin female should have a radius of 5–6 m. This was confirmed by Hirooka (1981) by means of direct observation of zigzag flight behavior of males towards virgin females, giving $R_A \cong 6$ m. Recently, Zhang et al. (1996) also reported a crude estimate of R_A as > 4 m by observation of males approaching synthetic pheromone.

In this study, we wanted to find the slope of a linear or linearized function with intercept m and slope b for the dependence of recapture y on distance x:

$$y = m - b \cdot x$$

From the parameters of such a regression the maximum R_S will equal the intercept with the x-axis. For the x-intercept one may calculate a confidence interval but this interval will be asymmetric and requires a nontrivial calculation (Sokol and Rohlf, 1981). As shown by Schlyter (1992), functions of the action of odor sources over distance are often nonlinear, and thus we will get a probability function of recapture, $P(R)$, over distance, R such that

$$P(R) = \text{retransformation function } (m, b)$$

Such a 1D function will then allow calculating a circular sampling area (2D) by integration to give the area, A, with sampling efficiency of unity, as suggested by Turchin and Odendaal (1996):

$$A = 2\pi \int_0^{R_S} R \cdot P(R)\, dR$$

Such an area may then be directly compared to areas of other sampling methods, such as larval web counts, which by definition have a unity sampling efficiency over their whole area.

In this paper, we estimate by mark-recapture the maximum range of sampling (R_S), and the corresponding sampling area for the fall web worm moth using a synthetic phèromone.

MATERIALS AND METHODS

Materials

Tests were carried out at Lizifang, Zhuanghe county, Liaoning province, P.R. China, from 1993-05-25 (first generation 1993) to 1994-07-31 (second generation 1994). A soybean field with an area more than 500 × 1000 m was chosen as the test site. Nonsticky Uni-traps (with DDVP as the poison) were used in the tests (see Zhang et al., 1996). The three Uni-traps were each baited with a synthetic pheromone dispenser, "92A" from Nitto Denko Co. (3Z,6Z)-(9S,10R)-*cix*-9,10-epoxy--3,6,-henicosadiene, (9Z,12Z,15Z)-9,12,15-octadecatrien-1-al, and (3Z,6Z)-(9S,10R)-*cis*-9,10-epoxy-1,3,6-henicosadiene, in a 1 : 10 : 1 ratio, total 6 mg/dispenser, (Zhang et al., 1996) were set up on bamboo poles 1.8–2.0 m high, in a line 15 m apart perpendicular to the prevailing main wind direction. New emergent male moths, usually collected at 6:00–6:30 PM, were marked with different fluorescent colors on the pronotum and released from various distances (30–250 m) downwind of the trap line. Wind speed was measured ≥3 times per night with a simple rotating cup anemometer.

The distance from the release point to the two off-center traps (the hypotenuse) is somewhat longer than the distances (the opposite) to the central trap (Fig. 1A). The distance, 15 m, between central trap and the off-center trap (the adjacent) is small compared to most distances (the opposite ranges 30–250 m). From the laws of sines and tangents it follows that *hypotenuse* = *opposite*/sin(atan(*opposite/adjacent*)) which simplifies to *hypotenuse* = $15\sqrt{1 + opposite^2/15^2}$. Only for opposite = 30 m is the difference >10% (hypotenuse = 33.5 m), but the exact distances have been used in the regression calculations. The difference in the calculation of the R_S using the inexact value is >10% but for the upper (outer) 95% confidence limit of R_S the error was as much as >50%. Statistical analysis was done with SPSS/PC+™ 5.0.1 (SPSS Inc., Noursis 1990). The intercept and 95% C.L. for the regression were obtained both by the regression facility of AXUM® 3.0 (Trimetrix Inc.) accessed via SPSS and by calculation following the procedure in Sokal and Rohlf (1981).

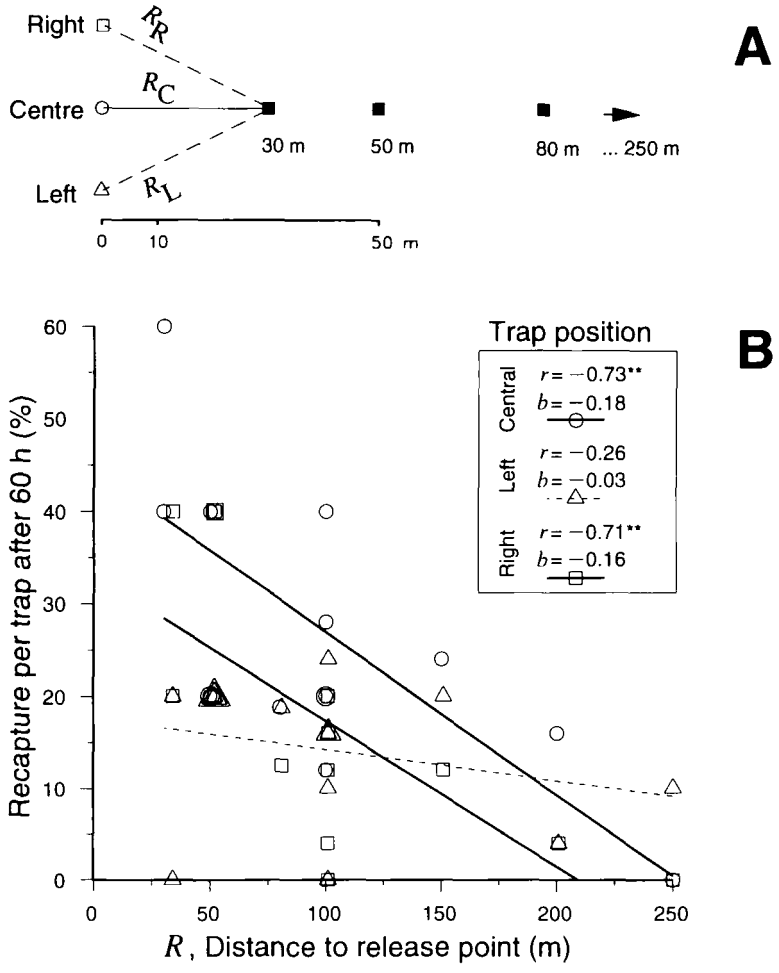

FIG. 1. (A) Experimental layout drawn to scale. (○, △, □) Pheromone traps, (■) Release platforms. R_R, R_C, and R_L) The range from a platform (30 m only drawn) to right, central, and left trap, respectively. The R_R and R_L are $10\% > R_C$ for $R_C = 30$ m. The exact values were used for x-values in regression estimates. (B) Recaptures per trap of *H. cunea* males. For regression lines in correlations: ----) $P > 0.05$ (N.S.), ——) $P < 0.01$ (**). Correlations were made on untransformed data, thus P-value levels are only rough indicators. The central trap position had higher catches than the left trap, while the decline of recapture over distance did not differ (slopes not different, see Table 2). (r) the Pearson correlation coefficient, (b) the slope of the regression line. Each differently sized marker of the same type centered on the same point show one of several overlapping data points. For details on regression parameters, see Table 2.

Methods

For a summary of dates and numbers of males released, see Table 1.

First Generation 1993. At 7:30 PM of May 25, marked males were released from 30 m (10 ♂♂ marked with red color), 50 m (10 ♂♂ marked with blue color), and 100 m (10 ♂♂ with black color) downwind of the trap line. The three Uni-traps were inspected at 7:30 AM on three consecutive days (at 12, 36, and 60 hr after release). The recaptured marked males were removed and counted. The same experiment was repeated once from May 28 to 31, except for marking color (purple, green, and yellow). The wind speed during the possible flight period (i.e., early evening, Zhang et al., 1996) was 1.5–2.5 $m \cdot s^{-1}$.

Second Generation 1993. At 7:30 PM of August 1, 25 ♂♂ marked with black color were released only from 100 m downwind of the trap line, as few newly emerged male moths were available at that time. The traps were checked 12, 36, and 60 hr after release. Wind speed was 0.5–1.0 $m \cdot s^{-1}$ in early morning, when second generation males fly (Zhang et al., 1996).

First Generation 1994. At 7:30 PM of May 17 and 21, 5 and 16 newly emerged ♂♂, marked with black were released from 50 m to 80 m downwind of the trap line, respectively. On May 25, 25 ♂♂ marked with red were also

TABLE 1. BASE DATA FOR MARK-RECAPTURE EXPERIMENTS WITH THE FALL WEB WORM MOTH, *Hyphantria cunea* (DRURY) IN LIZIFANG, LIAONING PROVINCE, NE P.R. CHINA, 1993–1994

Period/date	Males released per distance	Distances tested (m)	Recaptured males 12	36	60 hr	Total recapture (%)
First generation 1993						
May 25	10	30, 50, 100	22	1	0	100, 80, 50
May 28	10	30, 50, 100	22	0	0	80, 100, 40
Second generation 1993						
August 1	25	100	8	2	0	40
First generation 1994						
May 17	5	50	4	0	0	80
May 21	16	80	7	1	0	50
May 28	25	100	6	3	1	40
Second generation 1994						
July 24	25	100	16	1	0	68
July 26	25	150	13	1	0	56
July 28	25	200	4	2	0	24
July 31	10	250	1	0	0	10
All periods	Σ 176		Σ 103 ΣΣ 115	11	1	$\bar{x}$ 52.0

released from 100 m downwind. Trap monitoring for each distance was carried out at 12, 36, and 60 hr after release. The wind speed during the flight period (early scotophase) ranged 1.5–2.5 $m \cdot s^{-1}$.

Second Generation 1994. At 7:30 PM of July 24, 26, 28, and 31, 25 ♂♂ were marked and released from 100, 150, 200, and 250 m, respectively, downwind of the trap line, using a different color each day. Traps were monitored as above. Wind speed during the possible flight period (early morning in second generation) ranged from 0.4–0.9 $m \cdot s^{-1}$.

RESULTS

Recaptures per Trap and Night

Rate of recapture gives the basis for estimate of the range of action of a pheromone source. Recapture rates were high, in total 115 of 176 (65%) of the males released were captured. Rates were above 50% in 6 of 10 experiments (minimum 10%, maximum 100%) (Table 1). Total recapture rates were 40–100% over distances 30–150 m. Also at 200 and 250 m recaptures were 10–24% (Table 1). More than 90% of captures took place during the night of release for the first generation and in the next early morning for the second generation. For individual traps, recaptures after 12 hr were on average 18.1 ± 14.4% (±standard deviation SD, $N = 42$), while during the second night (36 hr) recapture was lower by a factor of ten at 1.2 ± 2.5%, and another decadic step lower on the third night (60 hr) at 0.10 ± 0.62%. In total, 103 of 115 (89.6%) males were recaptured during the first night (Table 1). No unmarked males of *H. cunea* and no other moth species were captured.

Rates of recapture are expected to vary with season due to uncontrollable factors such as ambient temperature or moth quality. However, in this work recaptures in different generations and years showed little difference (Table 1). In a 2-way ANOVA the result was $P > 0.2$ for both generation and year ($F < 1.8$, $df = 1,38$ for factors; $P = 0.97$, $F = 0.002$, $df = 1$ for the interaction). The central position of the three trap positions had a somewhat higher catch (ANOVA $P = 0.054$, $F = 3.14$, $df = 2,39$). More interestingly, the slopes of the regression lines on untransformed scales for recapture over distance (Figure 1B), did not differ among traps. There was no interaction of trap position × distance (ANOVA on transformed scales: $P > 0.1$, $F = 1.15$, $df = 12$) and the x-axis intercepts overlapped widely (Table 2).

There was a significant overall decline of recapture over distance ($r = -0.56$, $P < 0.01$**, $N = 42$, untransformed linear data), but the large difference between traps, with the central trap catching more (Figure 1B), made a regression based on the raw data points for each trap an imprecise estimate of R_S.

TABLE 2. SAMPLING RANGE (R_S) WITH 95% C.I. ESTIMATES BASED ON MARK RECAPTURES OF THE ARCTIID *H. cunea*

Recapture data	r	P (%)	(N)	SE estimate ($S_{Y \cdot X}$)	Y-intercept	Slope ($b_{Y \cdot X}$)	SE of slope (S_{b})	$\overline{Y}$	$\overline{X}$	N days	X-intercept (m)	95% Confidence Interval (m)
Individual traps after 60 hr												
Total %[a]	−0.56	0.01	42	1.58	32.0	−0.124	0.0294	19.5	100.4	2.5	257	202-404
p Central trap[b]	−0.80	0.06	14	0.131	1.04	−0.0548	0.0120	0.521	9.52	2.5	362	178-917
p Left trap[b]	−0.09	77.	14	0.177	0.400	−0.0049	0.0165	0.353	9.63	2.5	6700	NA-NA
p Right trap[b]	−0.76	0.49	14	0.152	0.936	−0.0577	0.0142	0.381	9.63	2.5	263	106-754
Aggregated per distance												
% recapture 60 hr[a]	−0.86	0.01	14	14.7	95.5	−0.369	0.0645	58.4	100.4	2.5	259	166-405
p recapture 12 hr[b]	−0.83	0.02	14	0.226	1.90	−0.118	0.0209	0.867	9.59	0.5	311	162-679
p recapture 60 hr[b]	−0.84	0.01	14	0.201	1.88	−0.102	0.0186	0.909	9.59	2.5	344	190-713
p recapture 60 hr[c]	−0.86	0.01	14	0.192	3.12	−1.148	0.197	0.911	1.93	2.5	523	207-2410

[a] Untransformed data.
[b] $\arcsin\sqrt{p} - \sqrt{Distance}$ regression.
[c] $\arcsin\sqrt{p} - \log(Distance)$ regression.

Recapture per Release Distance

As there was no interaction between trap position and distance, the data could be pooled for distance. When the catches of the three traps were summed to give one data point per release night and distance, a stronger correlation ($r = -0.856$, $P < 0.001$***, $N = 14$, untransformed data) was found between the total recapture proportion after 60 hr and distance, R. An approximately normal distribution of the dependent variable and an equidistantly spaced (uniform) independent variable was obtained by an arcsin $\sqrt{p}$ transformed catch (y) regressed on $\sqrt{R}$ (x) (Figure 2). The transformed data gave a slightly lower r-value (-0.844, $P < 0.001$***, $N = 14$) than untransformed data. As the x-values (R) are fixed and exactly known, the data allow for the use of a standard Model I linear regression (Figure 2). This type of regression is only a probability

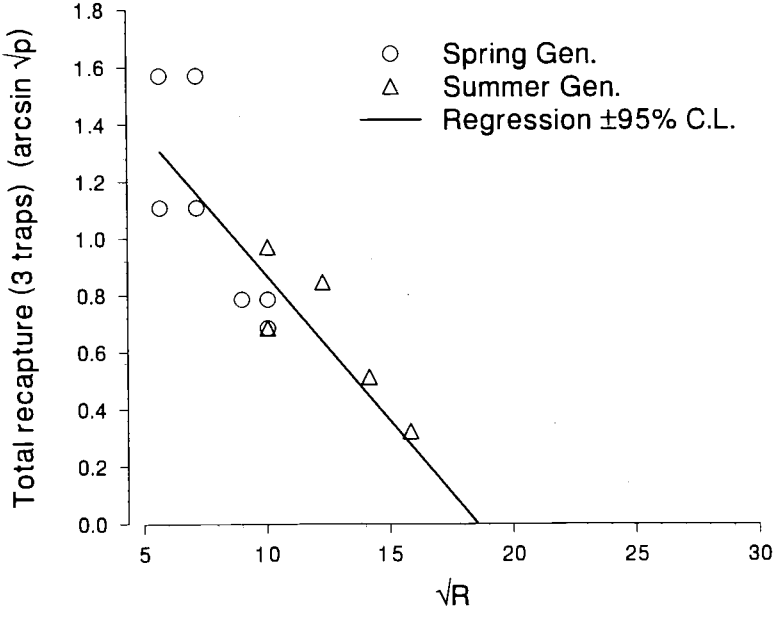

FIG. 2. Recaptures, summed for all 3 traps after 60 hr, of *H. cunea* males released from different distances down-wind a line of the traps. The x-intercept of the catch-distance regression lines corresponds to the maximum sampling range, R_S. Data and the regression line with its 95% confidence limits are displayed on the transformed scales. Regression of transformed y-values (proportion recaptured, in radians; arcsin $\sqrt{p}$) on transformed x-values (release distance, m: $\sqrt{R}$). Equation for regression (with ±SE of parameters): $y = 1.885(\pm 0.187) - 0.102(\pm 0.019)R$, $r^2 = 0.72$, $N = 14$. For more details on regressions parameters, see Table 2.

function (Turchin and Odendaal, 1996) for recapture over distance, but it gives directly the maximum sampling range (1 D), while the area sampled (2 D) requires an integration over the whole circle area to yield the sampling area.

The regression equation (with SE of parameters) for the total catch 60 hr after release was: $y = 1.88(0.19) - 0.102(0.018) \cdot x$, while after the first night at 12 hr it was:

$$y = 1.90\ (0.21) - 0.108(0.021) \cdot x\ (r = -0.83,\ P = 0.0002^{***},\ N = 14).$$

This gives a probability function of recapture, $P(R)$, over distance, R:

$$P(R) = (\sin(1.885 - 0.1016\sqrt{R}))^2$$

after sampling for 60 hr.

The regression allows a prediction of the maximum R_S (equivalent to the intercept with the x-axis) as 344 m with a confidence interval of 190–710 m (Figure 2). Regression of $\arcsin\sqrt{p}$ on $\log(R)$ gave a marginally larger r (-0.859), but gave a wider confidence interval for the intercept with the x-axis (Table 2). The first night of sampling with trap check after 12 hr gave an x-axis intercept close to the value for total catch after 60 hr (Table 2). The proportion of recapture at $y = 0$ (the x-axis intercept) corresponds to the maximum sampling distance (R_S) or the total area affected by the action of the sampling traps.

However, other probabilities of sampling can be estimated. The 0.5 intercept gives a 50% probability of recapture from 120 m (95% C.I.: 40–260 m).

Sampling Area

To get the area, A, where sampling efficiency is unity, we need to integrate the probability of sampling (here estimated as recapture probability, $P(R)$) from the source ($R = 0$) over each annulus of the circle area (dR) to the maximum sampling range (max R_S). The general equation is (modified from Turchin and Odendaal, 1996):

$$A = 2\pi \int_0^{R_S} R \cdot \mathrm{P}(R)\ \mathrm{dR};\ \text{and for our data we have:}$$

$$A = 2\pi \int_0^{\max R_S} R \cdot (\sin(1.885 - 0.1016\sqrt{R}))^2\ \mathrm{dR}$$

which for $R_S = 344$ gives 68,850 m^2 or 6.9 ha. If we would, naively, assume that the sampling efficacy is 100% all the way to the max R_S, the sampling area would be that of a circle with radius = R_S or 37 ha, a considerable overestimate by a factor of 5.4. Alternatively, the 6.9 ha corresponds more realistically to a circle with radius 148 m and sampling efficacy 100%.

DISCUSSION

The reliability of the data gathered here may be regarded as good, both in a biological sense–as recapture rates were consistently high and no difference existed between generations, and statistically–as correlations and regressions were highly significant. The r^2 values for both log and square-root transformed distance were >0.7. The confidence intervals were wide for the intercept with the x-axis (at $y = 0$, giving the maximum R_S), partly caused by the shortage of data points at high R values. This, in turn, depended on the unexpectedly high recaptures found at 100 m ($\geq 40\%$) which prompted investigation of the largest R values only in the latest experiments. Replication at the larger R values was as a result weaker. However, even the lower confidence limit for the maximum R_S is >100 m. As error margins or confidence limits for ranges of action of attractants are rare in the literature, comparisons with precision in earlier studies is hampered and we must still mostly compare estimates of central measures only.

Insects and attractants yield combinations that have widely different ranges of action. Schlyter (1992) said that "In general bark beetles seem to have smaller ranges than moths." However, variation within orders is large. While some Coleoptera such as *Tribolium castaneum* may have a range of attraction of about 2 m (Obeng-Ofori and Coaker, 1990), the curculionid *Cylas formicarius elegantulus*, the sweet potato weevil, has an R_S of at least 280 m (Mason et al., 1990) or as much as 470 m (recalculation by Schlyter, 1992). Even a kairomone response by a predatory beetle, *Teretriosoma nigrescens* Lewis (Histeridae), towards the synthetic pheromone of *Prostephanus truncatus* Horn (Col.: Bostrichidae) has a range of >100 m (Helbig et al., 1992). Some Lepidoptera such as *H. cunea* (this paper), *Cydia nigricana* (Wall and Perry, 1987) and *Chilo suppressalis* (Kondo and Tanaka, 1994), have R_S values >100 m. However, the Oriental peach moth *Carposina niponesis* (Oku et al., 1986) had an indirectly estimated R_S value of only about 20 m, and for the winter moth *Operophthera brumata* L. (Geometridae) Ambrus and Csoka (1992) found flight ranges of marked males to be only about 10 m.

The range of action observed here, longer than earlier reports for moths, could be explained by genetic, physiological, or ecological factors. Populations of an introduced and still spreading species may be selected to have a high frequency of good fliers. A species expanding its range during many generations would also benefit from characteristics that allow mate finding at initially very low population levels, such as high sensitivity of male peripheral and central systems (receptors, sensilla, sensilla frequency, neural pathways, CNS organization) or large amounts of female pheromone (as much as 1 μg/gland content in *H. cunea* reported by Hill et al., 1982). The large antenna of *Hyphantria* and

other arctiids may by themselves give better recaptures as they may act as a larger filter (more sensilla) for airborne molecules than those of the smaller tortricids. *H. cunea* is overall a large moth with good flight capability. Ecological factors, may however, be very important. The open area used, with little obstructions to flight, in combination with nonhost vegetation with no nearby natural pheromone sources will be optimum conditions for finding a source.

Zhang et al. (1997) suggested that "the insect density (web nests of *H. cunea* larvae) in (the coming) generation can also be predicted accurately . . . based on the trap captures" using the same trapping system as used here. For counting of larval webs on trees they used a circular area with radius 30 m. Recaptures at 30 m in our experiments in the open field were between 90 and 100%. The good ecological relation (trap catches–population dynamics) found by Zhang et al. (1997) could thus be anticipated from the behavioral data reported here, with a >90% capturing efficiency of males within the area sampled for larvae.

Our behavioral data might have overestimated R_S (due to the ecological factors discussed above), and the actual proportion caught from within 30 m might have been even higher in an area which had tall vegetation and many sources of natural pheromone (Zhang et al., 1997). Carter et al. (1992) found a poor relationship between male catches and level of defoliation at the leading edge of a gypsy moth *Lymantria dispar* (L.) infestation, which they suggested was due to a too large sampling area of traps compared to that of defoliation observations.

Population density may indirectly influence the range of sampling due to shorter wing lengths in high density populations with larval crowding (Carter et al., 1994). The present study was done in an open field with little physical obstruction of male flight and with no (known) competition with natural female pheromone sources from a resident population, as no unmarked males were found in our traps. Thus, sampling range could be expected to be smaller in a biotope with higher plants or trees and competing attraction sources.

Pheromone traps usually only function to attract the insects down-wind of a trap. Thus, sampling areas calculated by means of the whole circular area, as we simplistically did here, will be larger than the actual area of sampling if the wind direction is stable during the whole period of sampling.

Further studies on the sampling range in *H. cunea* should include tests in other biotopes and varying population levels. In addition replication at longer distances would be prompted by the present results.

The high recapture rates and the long R_S indicate that pheromone traps are highly efficient sampling devices for *H. cunea*. The R_S estimated here will be helpful as a first guide for trap placement in monitoring or mass-trapping schemes.

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ROLE OF ALLELOPATHY AS A POSSIBLE FACTOR ASSOCIATED WITH THE RISING DOMINANCE OF *Bunias orientalis L.* (BRASSICACEAE) IN SOME NATIVE PLANT ASSEMBLAGES

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Abstract—Leaf extracts of *Bunias orientalis* were shown to inhibit seed germination of a variety of cultivar plant species and of species cooccurring with *B. orientalis* in the field. Root exudate solutions and leaf litter leachates of *B. orientalis* were tested for their allelopathic activity using seedling growth assays. Additionally, in comparative seedling growth assays soil cores removed from dense *B. orientalis* stands were tested bimonthly for elevated allelopathic effects. The impact of root exudates on seedling growth was generally weak and varied between species. Similar results were obtained for the effect of *B. orientalis* leaf litter leachates on seedlings grown in sand culture relative to the effect of leaf litter leachates of a plant species mixture. When soil as a growth substrate was used, no consistent differences in seedling growth were obtained between the two litter leachate treatments. In the soil core experiment seedlings grown in soil cores collected from a dense *B. orientalis* stand unexpectedly showed better performance than seedlings grown in soil cores collected from a nearby mixed plant stand without *B. orientalis*, at least in early spring and late autumn. Predominating nutrient effects are, therefore, assumed to conceal a potentially increased allelopathic effect of soil beneath dense *B. orientalis* stands. It is concluded that other factors than allelopathy must be investigated to explain the rapid establishment of dense stands of this alien plant species.

Key Words—Allelopathy, *Bunias orientalis*, root exudates, leaf litter leachates, soil cores, growth inhibition.

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INTRODUCTION

Recent research has demonstrated allelopathic potential for several aggressive colonizer plant species, many of them being serious weeds (e.g., Weston et al., 1987; Li et al., 1992; Inoue et al., 1992; Yamane et al., 1992a, 1992b; Dolling et al., 1994). However, for some other potent weedy species previously suspected to be allelopathic, no considerable phytotoxic activity could be found, at least in experiments designed to be pertinent to evaluation of natural field conditions (Horsley, 1993; Choesin and Boerner, 1991).

Bunias orientalis L., a perennial Brassicaceae probably native to the southwest of Russia and now naturalized as a weed in most parts of Europe (Tutin et al., 1993) belongs to this group of potent colonizer plants, spreading rapidly in some regions of Central Europe in recent years (Ullmann et al., 1988; Steinlein et al., 1996). Thus, the question arises as to whether the rapid expansion of *B. orientalis* stands is promoted by allelopathic effects.

B. orientalis leaf extracts have been shown to considerably inhibit germination of standard (cultivar) and weedy species in petri dish bioassays. A chemical analysis of these extracts yielded cinnamates and norisoprenoids as possible candidates for this allelopathic effect (Dietz and Winterhalter, 1996). Such bioassays certainly are of value for tests of the allelopathic potential of organisms (Leather and Einhellig, 1986), but often they are not suitable to provide evidence for allelopathic effects in the field (Stowe, 1979; Heisey and Delwiche, 1983; Lawrence et al., 1991). In this respect it is crucial to investigate whether 'natural' pathways of egression of the possible allelochemics (e.g., by root exudation or by leaching of living or decomposing leaves) into the respective growth substrate lead to phytotoxic effective concentrations for receptor plants grown therein. Additionally, investigations of the rhizosphere soil obtained from the field site may be useful for the determination of phytotoxic plant–plant interactions in the field (e.g., see Henn et al., 1988; Li et al., 1992; Horsley, 1993).

The aim of this present study was to determine (i) whether exudates of *B. orientalis* roots impair growth of seedlings in sand culture, (ii) whether leachates from decaying *B. orientalis* leaves show elevated allelopathic effects on seedlings growing in sand or soil as compared to leachates from a plant species mixture and (iii) whether soil collected from dense *B. orientalis* stands inhibits seedling growth relative to soil collected from a nearby plant stand without *B. orientalis* due to possible accumulation of allelochemics, and to determine if there are seasonal variations in the corresponding results.

MATERIALS AND METHODS

Donor and Receptor Plants. For all greenhouse experiments *B. orientalis* donor plants were grown from fruits collected the year before the study at a

location with dense *B. orientalis* stands in the vicinity of Würzburg (Germany). Most of the chosen receptor (indicator) plants are widespread nitrophilic species actually or potentially cooccurring with *B. orientalis*: Seeds of *Lactuca sativa* L. var. Kagraner Sommer/Duna, *Hordeum vulgare* L., *Arctium tomentosum* Mill., *Artemisia vulgaris* L., *Cirsium vulgare* (Savi) Ten, and *Rumex obtusifolius* L. were obtained from Carl Sperling & Co. (Lüneburg, Germany); seeds of *Arrhenatherum elatius* (L.) J. et C. Presl and *Tanacetum vulgare* L. were collected at a nearby field site the year before the study.

Petri Dish Bioassay. In this study we concentrated on perennial receptor species cooccurring with *B. orientalis* in the field. Therefore, at first it was necessary to determine whether they exhibit susceptibility to allelochemics from *B. orientalis* as was previously shown for the cultivar species *L. sativa* and *H. vulgare* and the annual herb *Matricaria inodora* L. (Dietz and Winterhalter, 1996). For that purpose petri dish bioassays were performed to test for germination inhibition for each indicator species. Twenty five seeds each of *T. vulgare*, *C. vulgare*, *A. vulgaris*, and *R. obtusifolius* were sown on filter paper discs within petri dishes (five replica plates each) and were added with 3 ml of *B. orientalis* leaf extract prepared by immersion of leaves in distilled H_2O (20% w/w, 3 d) or distilled H_2O only (control), respectively. Germination percentage was measured after 100 hr in the growth chamber. Additionally, for *R. obtusifolius* showing rapid and complete germination, radicle lengths were measured to the nearest 0.5 mm. *A. tomentosum* and *A. elatius* were excluded from the test due to their prolonged germination behavior. For further details and a discussion of osmotic effects see Dietz and Winterhalter (1996).

Root Exudate Recirculating System. A bioassay of seedling growth inhibition by phytotoxic root exudates was performed following the root exudate recirculating method of Stevens and Tang (1985) in a modified design. Plastic pots of 17.5 cm height, 20 cm diameter (volume 4 l) were used as donor pots and as acceptor pots. Hose unions were fixed centered at the bottom of the pots and connected to plastic tubes with an inner diameter of 1 cm for circulation of nutrient solutions. All tubes were wrapped with aluminium foil to exclude light. Donor and acceptor pots of a recirculating system were set up in a staircase arrangement. Two boards with drilled holes allowing vertical passage of tubes, were used. The board supporting the acceptor pots was lifted 40 cm relative to the board supporting the donor pots, thus enabling nutrient flow from acceptor pots to donor pots by gravity. Nutrient solution flow back to the acceptor pots was managed by means of an electric air pump (flow rate 1.5 liters/hr). The bottoms of the pots were covered with cheesecloth to retain suspended matter from entering the tubes. The cheesecloth was topped with a 5 cm layer of porous clay pebbles (Lecaton, Germany). The rest of the pot volume was filled with coarse grade silica sand (0.7 mm grain size).

To every recirculating system 2.75 l of a modified Hoagland nutrient solution was added once, which was supplemented with ammonia and iron (Smith

et al., 1983). Concentrations of macronutrients were (mmol/l): KNO_3 8.0, $Ca(NO_3)_2$ 3.0, NH_4Cl 5.0, NaH_2PO_4 1.0, $MgSO_4$ 2.0 and Fe-EDTA 0.1. Nutrient levels were monitored using an anion analysator (Biotronik, Germany) and test sets for cations and were maintained by selectively replenishing exhausted salts (mostly phosphate and nitrate). Water loss due to evapotranspiration was determined every day by weighing both pots of each system with a spring-balance (precision $\pm$ 100 g). The lost amount of water was replenished daily. The pH of the nutrient solutions was constant at 6.0 during all experimental runs.

The experiments were performed in a glasshouse with temperature regulation (25°C/10°C daytime maximal temperature/nighttime minimal temperature) and ambient light conditions (but excluding direct sunlight between April and October 1994).

For each indicator species (*L. sativa*, *H. vulgare*, *A. elatius*, and *A. tomentosum*) one experiment was performed using three replicate treatment systems (*B. orientalis* as donor plant, 100% nutrient strength) in parallel with two triplicate control systems (no plants in donor pots, 100% or 60% nutrient strength, respectively). The recirculating systems were randomly assigned to the nine positions on the boards. For the treatment systems vegetative *B. orientalis* plants (mean rosette leaf length of about 25 cm) were removed from the culture pots, the root systems were gently rinsed, and four plants each were then planted into the donor pots. Donor pots of the control systems without *B. orientalis* plants were inoculated with about the same amount of soil which was introduced to the treatment systems adhering to *B. orientalis* roots. For each new experiment the silica sand was completely replaced, the clay pebbles thoroughly washed, and the tubes were either replaced or kept in boiling water for about one hr prior to reuse.

Three days after the start of nutrient circulation a surplus of seeds was sown into each of the receptor pots and covered with a 0.5 cm layer of silica sand. Upon emergence seedlings were thinned to 30 equally sized seedlings/pot. After 12 to 18 days, depending on the receptor species, the seedlings were harvested, shoot and root lengths measured, and dry weights were determined after oven-drying at 70°C for 24 hr. As *L. sativa* seedlings showed high mortality, only proportions of dead, damaged, and vital seedlings, respectively, were determined.

Leaf Litter Leachates. A comparative bioassay of seedling growth inhibition by leaf leachates was performed in the glasshouse (conditions see above) by comparing the effects of *B. orientalis* leaf litter on seedling growth with those of leaf material of a plant species mixture. Six flats (30 × 20 × 5 cm length/width/height) were filled with coarse grained silica sand and another six flats were filled with loamy soil. Leaf material from *B. orientalis* and several cooccurring species was freshly harvested at a location near Würzburg. For both substrates *B. orientalis* leaves (50 g f.w. per flat) were spread on the three flats

each. The remaining six flats were prepared with leaf material (50 g f.w. per flat) from the species mixture: *Artemisia vulgaris* (7.5 g), *Solidago canadensis* L. (11 g), *Heracleum sphondylium* L. (5 g), *Geranium pratense* L. (19 g) and grasses (7.5 g). During the first week the flats were kept wet by spraying them with 0.5 ml/l Wuxal (12 Vol % N, 4 Vol % P, 6 Vol % K) fertilizer solution (flats containing soil) or 1.0 ml/l Wuxal NPK 12-4-6 fertilizer solution (flats containing sand), respectively. Thereupon, all flats were kept moist using tap water. The amount of fluid added was adjusted depending upon evaporation such that the leaves and substrates remained moist all the time to speed up the decomposition process but avoiding leakage through the bottom of the flats. After 12 days the decomposed leaves were moved to the rims of the flats to form a ring. Within this ring a surplus of pregerminated seeds of *A. elatius*, *C. vulgare*, *R. obtusifolius*, and *A. tomentosum* was evenly planted in each flat and slightly covered with the respective substrate. Upon emergence the seedlings were thinned to yield about ten evenly distributed seedlings per species and flat. The seedlings were harvested after a growth period of two weeks, prior to apparent resource competition (mutual overshading). Seedling height, root length, and dry weight (after oven-drying at 70°C) were determined for the flats containing sand. For the flats containing soil, root length was omitted and dry weight was measured for above ground biomass only.

Soil Core Experiment. In order to test for seasonal enrichment of allelochemics in the soil beneath dense *B. orientalis* stands, soil cores (∅ 7 cm, depth 10 cm) were collected at two adjacent sites in the vicinity of Würzburg, one with dense cover (80–90%) of *B. orientalis* and the other with an equally dense mixed plant stand without *B. orientalis* about 20 meters apart (control). The soil type was loamy clay at each site. On March 28, 30 soil cores from each site were collected. The same day, each soil core was transferred into a plastic pot with free water drainage (∅ 8 cm, height 9.5 cm) without mixing up the stratification of the soil. Every pot was planted with four pregerminated seeds of one of the following species: *R. obtusifolius*, *C. vulgare*, *A. tomentosum*, *T. vulgare*, *A. vulgaris*, and *A. elatius*. Five replica pots were used for each species x treatment (site) combination. The seedlings were grown in a growth chamber for four weeks. Light and temperature were regulated as follows: 500 μmol/m^2/sec photon flux density (metal halide lamps); 14 hr light period with 22°C and 10 hr dark period with 10°C. The pots were watered daily with distilled water. Regenerating shoots and seedlings of preexisting species were clipped at soil surface level. The height of the seedlings (*C. vulgare*: rosette diameter) was measured weekly and above ground dry weight (70°C) was determined at harvest. Starting in March, the soil core removal and ensuing seedling growth experiment were repeated every two months until November. The July data set had to be omitted from the analysis due to disproportionate watering of treatment and control pots.

Statistical Analyses. All analyses were run using STATISTICA for Win-

dows (Statsoft, Inc. 1995, Tulsa, OK) with values of $P < 0.05$ accepted as significant.

One factor multivariate analysis of variance (MANOVA) was used to test for overall differences in the root exudate recirculating bioassays employing root length, shoot length, and dry weight of the respective seedlings as dependent variables. If this test was significant, univariate F-tests for each variable were examined to interpret the respective effects. For each variable with significant effect post hoc comparisons (Scheffé-test) were performed to determine the factor levels (*B. orientalis* systems, 100% control systems, 60% control systems) with significant differences.

For the leaf litter leachate experiment MANOVA was used separately for each seedling species. To avoid capitalizing by chance, a Bonferroni-adjusted significance level of $\alpha = 0.0125$ was used (Sokal and Rohlf, 1995). Upon significant results univariate F-tests again were performed for investigation of main effects.

For each experimental run of the soil core experiment, differences between soil core treatments, expressed as above ground dry weight and seedling height at harvest time were tested by using two-way analysis of variance (ANOVA, site x species design). This way the overall site main effect could be tested apart from the species effect. For each significant effect *t*-tests for each seedling species were performed regarding seedling height and dry weight to find out which species contribute to the significant differences between soil cores at the respective seasons. For these tests Bonferroni adjustment was used ($\alpha = 0.0083$).

RESULTS

Petri Dish Bioassay. For all species tested *B. orientalis* leaf extracts showed considerable germination inhibition (Table 1). For *R. obtusifolius* this effect was not obvious from the percentage of germinated seeds but from the greater radicle lengths in the control dishes.

Root Exudate Recirculating System. In general, *B. orientalis* root exudates showed only weak, though significant impact on seedling growth for most of the tested species. For *L. sativa*, the most sensitive species in the test runs, 70% of the seedlings were dead by harvest time and the rest showed leaf necroses in the treatment systems. In the control systems, about 30% of the seedlings remained vital, roughly 30% were damaged and a distinctively lower proportion of seedlings died (45% or 31% in the 100% or 60% control systems, respectively). *H. vulgare* seedlings in the treatment systems showed considerable shorter shoots and roots (by about 20% and 35–40%, respectively) as compared to the controls ($P << 0.001$, Figure 1 A). The comparison between the 60% and 100% nutrient strength controls revealed only minute though significant differences ($P = 0.047$ and 0.019, respectively) due to low variability in this

TABLE 1. INHIBITORY EFFECTS OF *B. orientalis* LEAF EXTRACT ON SEED GERMINATION OF FOUR DIFFERENT INDICATOR SPECIES (PETRI DISH BIOASSAY)

	Germination [%]	
Species	Extract	Control
T. vulgare	0	23
C. vulgare	0	8
A. vulgaris	1	15
R. obtusifolius	91	97
	Mean radicle length [mm]	
Species	Extract	Control
R. obtusifolius	"*p*" (78%), 0.5 (22%)	2.5

"*p*" = radicle protruding.

species. The dry weight was reduced to a lesser extent ($P < 0.05$, Figure 1 B) for the treatment pots. In this case there was no difference between the controls ($P = 0.473$). For *A. elatius* shoot and root lengths were not significantly different between the treatment and control systems ($P = 0.084$ and 0.563, respectively, Figure 1 C). As compared to the 60% controls, dry weights in the treatment systems as well as the 100% controls were slightly but significantly reduced ($P < 0.001$ and $P = 0.024$, respectively, Figure 1 D). But there was no difference between treatment and 100% controls ($P = 0.325$). Thus, differences in dry weight for *A. elatius* in this case might be attributed mainly to nutrient effects. *A. tomentosum* dry weight was significantly reduced (by about 20%, Figure 1 F) for the treatment systems in comparison to both controls ($P < 0.05$), whereas shoot length showed only marginally significant differences between the treatment and the controls ($P < 0.09$ in both cases). Root lengths were not significantly different ($P = 0.728$).

Leaf Litter Leachates. The overall treatment effect for seedlings grown in flats filled with sand was significant for shoot length and dry weight ($P = 0.033$ and 0.002, respectively), i.e., on average seedling growth was slightly impaired in the *B. orientalis* treated flats as compared to the flats treated with litter of the species mixture (first column in Figure 2). If tested for each species alone, only *A. tomentosum* showed a considerable contribution with marginal Bonferroni-adjusted significance ($P = 0.019$). Here, both shoot lengths and dry weights were reduced by about 25% ($P = 0.002$ and 0.022, respectively). Though not significant if tested separately ($P > 0.2$), the other species further contributed to between-treatment dry weight variance as indicated by the *P*-value (0.002) in the overall test.

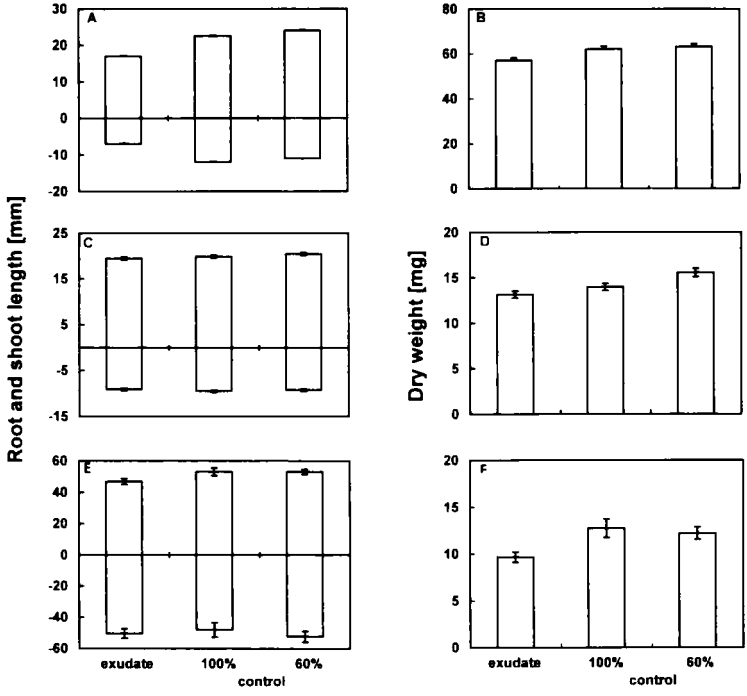

FIG. 1. (A-F) Root and shoot lengths and dry weights of receptor species seedlings grown in *B. orientalis* root exudate solution as compared to the controls. (A, B) *H. vulgare*; (C, D) *A. elatius*; (E, F). *A. tomentosum*. Error bars indicate ± S.E.

No clear growth differences could be obtained from seedlings of *B. orientalis* treated soil flats as compared to soil flats with species mixture leaves (second column in Figure 2). For the soil flats an overall treatment effect was found for dry weight ($P = 0.006$) but not for shoot length ($P = 0.557$). Again, this effect was caused by *A. tomentosum*, which showed a significantly higher dry weight in *B. orientalis* treated flats ($P = 0.003$). All other species neither showed significant differences in dry weight ($P >= 0.05$) nor a consistent trend for this variable for further explanation of between-treatment variance.

Soil Core Experiment. The results of the soil core seedling growth experiment exhibited considerable seasonality. On the whole, seedling growth in soil cores collected in early spring (March) and in late autumn (November) was considerably higher in the samples obtained from the dense *B. orientalis* stand as compared to the non-*B. orientalis* one (Figures 3 and 4). Except for *R. obtusifolius* and *T. vulgare* in the months between, i.e., May and September

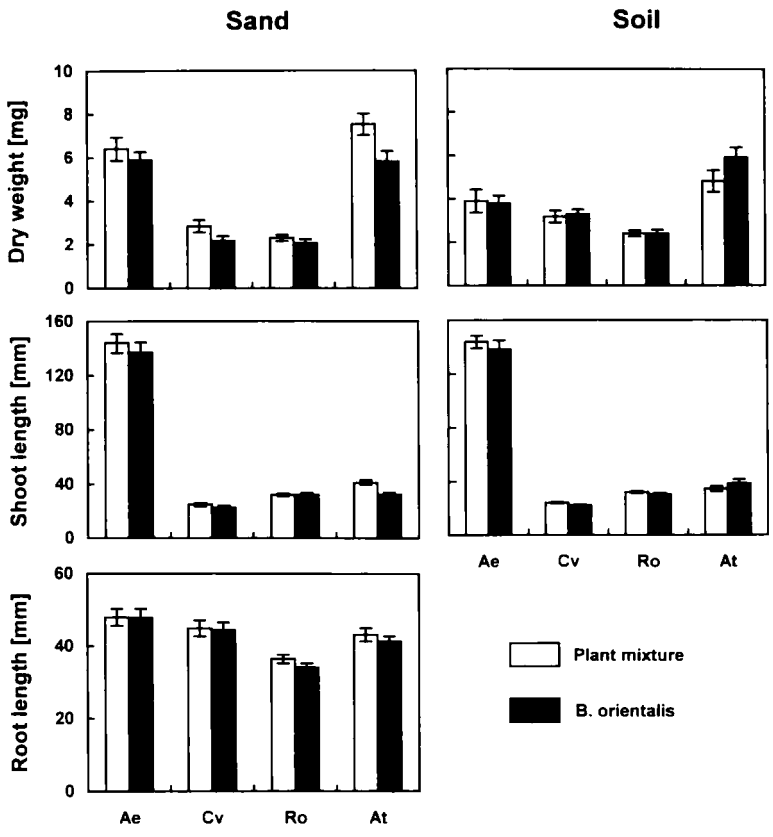

FIG. 2. Results of the leaf leachate experiment. Root lengths, shoot lengths, and dry weights of indicator species seedlings grown in sand culture and soil culture, respectively. Ae = *A. elatius*; Cv = *Cirsium vulgare*; Ro = *R. obtusifolius*; At = *A. tomentosum*. Error bars indicate ± SE.

(July was omitted from the analysis) there were no such prominent growth differences between both treatments (Figure 3).

In March, seedlings showed significantly higher shoot lengths and dry weights in *B. orientalis* soil cores ($P < 0.001$, Figure 3). *C. vulgare* and *A. elatius* differentiated strongly between treatments for both growth parameters ($P < 0.005$). *A. vulgaris* shows marginally significantly better growth in *B. orientalis* soil cores ($P < 0.025$). The other species, except *T. vulgare*, showed no significant differences ($P > 0.08$) though the trend was the same. In May, no significant differences could be observed between the treatments ($P = 0.485$,

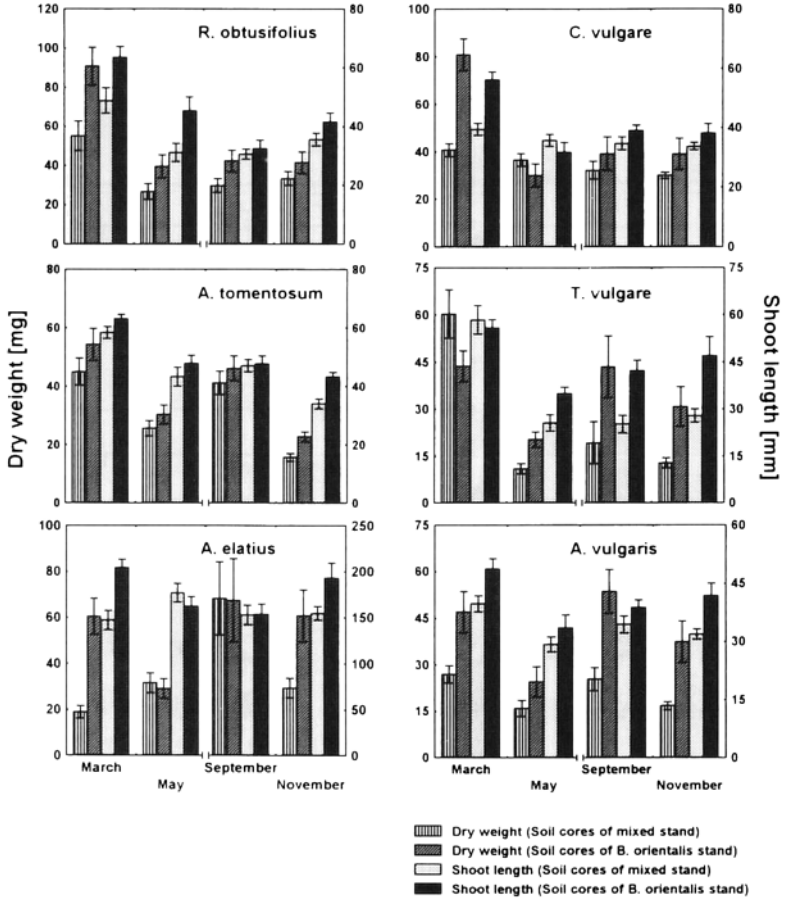

FIG. 3. Results of the soil core experiment. Shoot lengths and dry weights of indicator species seedlings grown in soil cores removed from a dense *B. orientalis* stand or from a mixed stand without *B. orientalis*. Shoot length data are from the measurement at harvest time. Error bars indicate ± SE.

Figure 3). In September, there was again a significant trend for better growth in *B. orientalis* soil cores concerning dry weight ($P = 0.015$) but not for shoot length ($P = 0.068$). No species contributed significantly to this trend when tested separately ($P > 0.03$). In November, performance of seedlings grown in the *B. orientalis* soil samples again were clearly better than in the soil samples from the non-*B. orientalis* stand ($P < 0.001$, Figure 3). This time, *A. tomen-*

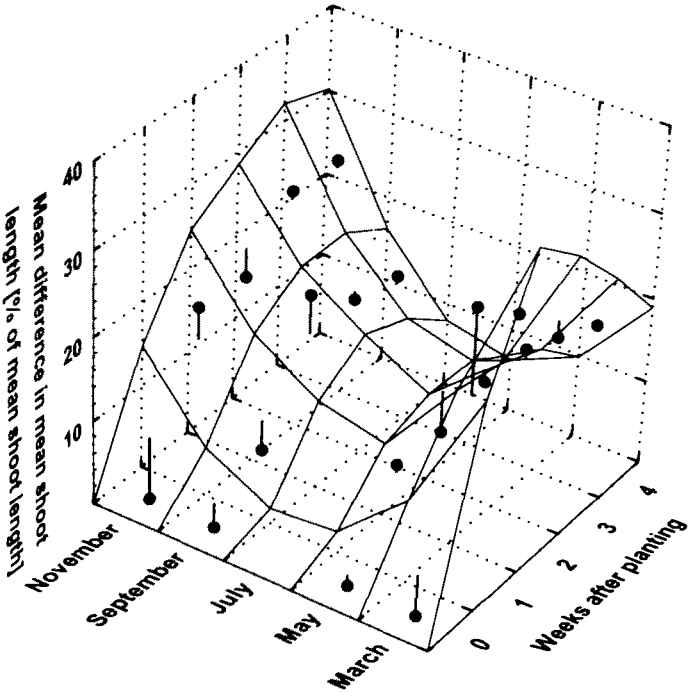

FIG. 4. Time course of differences in shoot lengths between seedlings grown in soil cores removed from a dense *B. orientalis* stand and seedlings grown in soil cores removed from a mixed plant stand. Differences were standardized (% of species mean shoot length) and data were generated by averaging the standardized differences of all six species tested. A spline smoothed surface is fitted to the data points to elucidate the general trend.

tosum and *T. vulgare* contributed most to the observed effect ($P < 0.002$), but also *A. elatius* dry weight ($P = 0.004$) and *A. vulgaris* growth ($P <= 0.01$) were significantly or marginally significantly higher in *B. orientalis* soil cores.

DISCUSSION

Root exudates from *B. orientalis* showed significant inhibitory effects for three out of the four species tested. Pronounced impact could only be observed for shoot and root development of *H. vulgare* and for mortality of *L. sativa*. These species are frequently used as indicator species for bioassays (e.g., Heisey and Delwiche, 1983; Heisey and Delwiche, 1985; Leather and Einhellig, 1986;

Barnes et al., 1986; Li et al., 1992) and therefore, may be predisposed for comparative studies. On the other hand, as cultivated species *L. sativa* and *H. vulgare* might be particularly susceptible to allelochemics compared to other species (see Stevens and Tang, 1985; Yun and Kil, 1992). The two remaining species, *A. tomentosum* and *A. elatius*, which are widespread species naturally cooccurring with *B. orientalis*, showed no or only weak response to root exudates. Of course, the results of our experimental design are limited to evidence of phytotoxic compounds exuded by roots of first-year vegetative plants, but considering the densely packed growth of four of them, in a system with small substrate volume, concentration of exuded allelochemics can be supposed to be higher than in dense *B. orientalis* stands. Moreover, the results of the leaf litter leachate test (see below) suggest that the efficiency of the allelochemics is diminished in (loamy) soil relative to sand substrate. Thus, in the face of our study, a considerable effect of root exudates for allelopathic growth inhibition in the field seems to be very unlikely.

The amount of leaves used for the leaf litter leachate assay (50 g d. w. per 600 cm^2 of substrate area) roughly corresponds to decomposition of 25% of total above ground leaf biomass of dense *B. orientalis* stands (unpublished data). Therefore, considering the shallow substrate layer (thickness 5 cm) of the flats and the prevention of leakage through their bottoms, it is reasonable to assume the concentrations of leached allelochemics in the respective substrates in the flats to be comparable with the conditions at the field site, provided that the decomposition rate of allelochemics *in situ* is sufficently high to prevent marked accumulation (see Rice, 1984).

Decaying leaves of *B. orientalis* weakly reduced seedling growth as compared to decaying leaves of a species mixture when sand was used as a substrate. In the soil design only species-specific growth differences differentiated between the two treatments without a consistent trend. This demonstrates the importance of the chosen substrate for the outcome of experiments aimed at evaluating the effects of allelochemics. On the other hand, a trade-off between beneficial effects of liberated nutrients and inhibitory effects of leached allelochemichs must be taken into account when interpreting the results (Li et al., 1992; Rutherford and Powrie, 1993). Therefore, evidence of the sand design suggests that the proportion of both effects is less favourable in the case of decaying *B. orientalis* leaves. As *B. orientalis* clearly shows no distinctly higher allelopathic effect arising from decomposing leaves compared to other coocurring species, particularly for soil conditions, this egression pathway of allelochemics, too, probably does not contribute to competitive advantage in the field.

The soil core experiment aimed at estimation of accumulation of allelochemics dependent on the season *in situ* yielded the unexpected result that not only no elevated allelopathic potential could be detected for soil from the *B.*

orientalis stand as compared to the non-*B. orientalis* stand during most of the vegetation period, but also soil conditions of the former were considerably improved for seedling growth in early spring and late fall. Obviously, particularly high nutrient input into the upper soil layer by mineralization of the great amount of above ground biomass typical for dense *B. orientalis* stands in autumn and early winter, may be responsible for this effect. Consistently, we observed improved soil conditions, i.e., higher contents of organic matter, in the sampled soil layer. Thus, a possible increase in allelochemics due to massive decomposition of leaf litter from *B. orientalis* seems to be overcompensated by far by remineralization or improvement of the nature of the topmost soil layer, respectively.

An important finding relevant for the initial questioning is that in our experiments considerable species specificity in reaction to the allelochemics or nutrient levels is apparent which was also found in previous research (e.g., Heisey and Delwiche, 1983; Rutherford and Powrie, 1993). Thus, in studies on allelopathy a variety of receptor species should be included to obtain general information about allelopathic effects of species under consideration, unless one is merely interested in effects on a certain target species (as for instance in agricultural research). Consequently, it may be apt to choose species naturally cooccurring with the investigated species in addition to more commonly used indicator species, when one targets at evaluation of the ecological importance of allelopathy *in situ*.

In summary, the results obtained within the scope of our study yielded no evidence for considerable allelopathic effects from *B. orientalis*. This distinctly contrasts the results from the petri dish germination bioassays and once again underscores the incongruity between allelopathic potential, as obtained by bioassays screening plant extracts, and the effectiveness of allelopathy under more natural conditions. Clearly, the conditions for duration, chemical modification, and availability for target plants of allelochemics in (loamy) soil (see Rice, 1984, chapter 14) is distinctly different from pure sand substrate or even more artificial substrates (e.g., hydroponic culture). Even if assumed that there be no distinct nutrient effects in the rhizosphere soil beneath dense *B. orientalis* stands, deposition of allelochemics caused by this species may either be rendered inefficient by the soil chemical reactor and/or may not be distinctly more efficient than deposition of allelochemics of the other cooccurring species.

Thus, and in the face of the at most weakly significant allelopathic effects observed in our study, our data did not support allelopathy as a substantial factor in the competitive ability of *B. orientalis*. We therefore, currently focus on growth patterns (Steinlein et al., 1996) and population dynamical aspects of *B. orientalis* to unravel the key traits underlying the rapid establishment and persistency of its stands.

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INTERCASTE, INTERCOLONY, AND TEMPORAL VARIATION IN CUTICULAR HYDROCARBONS OF *Coptotermes formosanus* SHIRAKI (ISOPTERA: RHINOTERMITIDAE)

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Abstract—We characterized the variation in cuticular hydrocarbon mixtures between seven colonies of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki, from the same population. We report differences between castes, between colonies, and within the population over time to assess seasonality. Colonies of *C. formosanus* from Oahu, Hawaii, were sampled for 25 months. Each month, one sample each of 200 workers, 50 soldiers, nymphs, or alates from each colony was subjected to gas chromatography-mass spectrometry (GC-MS) analysis of the cuticular hydrocarbons. We resolved 39 individual peaks and identified 52 individual or isomeric mixtures of hydrocarbons. Only *n*-alkanes and methyl-branched alkanes occur; no olefins were found. Internally branched monomethylalkanes were the most abundant class of hydrocarbons, representing 45% to 50% of the total. 9-;11-;13-Methylheptacosane accounted for over 30% of the total hydrocarbon for all castes. 2-Methyl- and 3-methylalkanes comprise approximately 30% of the total. Internally branched dimethylalkanes constitute 15% to 20% of the total cuticular hydrocarbon. Only one trimethylalkane, 13,15,17-trimethylnonacosane, was found in small amounts. The hydrocarbon mixtures of all four castes were similar. Quantitative differences in hydrocarbon mixtures among the castes were easily displayed using canonical discriminant analysis. Soldiers and workers are significantly different from one another and from nymphs and alates. Nineteen peaks are statistically significant between workers and soldiers. Nymphs and alates were not statistically different. We detected statis-

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tically significant quantitative differences between colonies in 18 peaks for workers and 12 peaks for soldiers. Each of the colonies of *C. formosanus* can be separated from the others by the proportions of their hydrocarbon components. We detected statistically significant differences between months of the year for 12 peaks for workers and four peaks for soldiers; two peaks each for workers and soldiers showed distinct, seasonal trends. This seasonal shift in proportions of hydrocarbons correlates with the production of alates.

Key Words—Formosan subterranean termite, *Coptotermes formosanus*, founder event, biogeography, populations, seasonal variation, Isoptera, Rhinotermitidae, cuticular hydrocarbons, Hawaiian insects.

INTRODUCTION

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is one of the most economically important subterranean termites species in the world and is a serious threat to wood in structures wherever it occurs (Su and Scheffrahn, 1990). Even though the common name implies that this species is from Formosa (Taiwan), Kistner (1985) considered *C. formosanus* to be indigenous to mainland China on the basis of its associated termitophiles. This species was introduced into Japan prior to 1600 and was first collected in Hawaii in 1907 (Swezey, 1914), although it may have been present as early as 1869 (Tamashiro et al., 1987). Since its introduction into Hawaii, *C. formosanus* has been reported to be established in Guam, Midway Island, Sri Lanka, Taiwan, South Africa, and the mainland United States (Su and Tamashiro, 1987). Morphological examination and characterization of the cuticular hydrocarbons of recent collections of *Coptotermes* from Guam, however, indicate that a species other than *C. formosanus* is present there (Su, 1994; Su and Scheffrahn, 1996; Haverty, unpublished observations). It is possible that two or more species of *Coptotermes* occur on Guam.

Infestations of *C. formosanus* were first noticed in the continental United States about 1965. It is likely, however, that colonies of *C. formosanus* were established soon after the end of World War II. Storage of *C. formosanus*-infested military goods at facilities near port cities of the United States has been implicated as the means of establishment of *C. formosanus* in North America (Beal, 1987). Once *C. formosanus* was firmly settled in locations such as New Orleans, Louisiana; Galveston, Texas; and Charleston, South Carolina, it began to appear in other port cities in the southeastern United States including Baton Rouge, Louisiana, and Hallandale, Florida (La Fage, 1987).

The analysis of cuticular hydrocarbon components of *C. formosanus* indicates that this species is separable from other species of *Coptotermes* characterized thus far on the basis of qualitative differences in cuticular hydrocarbons (Brown et al., 1990, 1994; Haverty et al., 1991, 1992). Quantitative differences

in cuticular hydrocarbons have been used to separate geographic populations of *C. formosanus* (Haverty et al., 1990a). Analysis of the cuticular hydrocarbons of workers from several colonies each from Hallandale, Florida; New Orleans, Louisiana; Lake Charles, Louisiana; and Honolulu, Hawaii suggests that *C. formosanus* was introduced into the United States numerous times (Haverty et al., 1990a). Populations from two geographically proximate locations, New Orleans and Lake Charles, displayed quantitatively different hydrocarbon mixtures, which strongly supports the hypothesis that at least two separate introductions were made into the state of Louisiana (Haverty et al., 1990a; La Fage, 1987). Since 1980, indications of established infestations in inland areas such as Auburn, Alabama; Meridian, Mississippi; Orlando, Florida; Atlanta, Georgia; and La Mesa, California, suggest this species may now be transported in infested wood products or potted plants via domestic commerce (Atkinson et al., 1993; Chambers et al., 1988; La Fage, 1987; Sponsler et al., 1988).

Allozyme electrophoresis has not yet proven to be a useful technique for investigating the distribution of *C. formosanus* populations in the United States. Korman and Pashley (1991) used standard starch gel electrophoresis to examine allozymic differentiation in colonies of *C. formosanus* from four populations (New Orleans, Lake Charles, Hallandale, and Honolulu). Very little variation was found. They found only limited differentiation in allele frequencies between populations, but concluded that mainland populations did not result from a single introduction.

Strong and Grace (1993) surveyed 13 colonies of *C. formosanus* in Hawaii and resolved 29 protein loci using cellulose acetate gel electrophoresis. Eight of these loci proved to be stable, but were found to be monomorphic across all colonies. Subsequently, preliminary results from starch gel electrophoresis confirmed the observation by Korman and Pashley (1991) of a low level of polymorphism at the glucose phosphate isomerase (Gpi) locus (Strong and Grace, 1993). Neither Korman and Pashley (1991) nor Strong and Grace (1993) were able to separate colonies or populations (several colonies from the same area) of *C. formosanus* by analysis of allozymes.

Similar conclusions were reached by Broughton and Grace (1994) when they surveyed mitochondrial DNA restriction sites from 10 colonies of *C. formosanus* from Hawaii. They discovered that there was no variation in mitochondrial DNA in any of the colonies they examined. Their results corroborate the findings of Korman and Pashley (1991) and Strong and Grace (1993) that *C. formosanus* populations in Hawaii, and on the U.S. mainland, have a very low level of genetic polymorphism, and suggest that this species was depauperate of genetic variation in comparison to the approximate 40% polymorphic loci for the Class Insecta as a whole (Nevo 1978). The survey of these geographically distinct colonies of *C. formosanus* on the Hawaiian islands of Oahu and Maui by Strong and Grace (1993) and Broughton and Grace (1994) raise

the possibility that the extremely low level of allozyme and mitochondrial DNA variation among these colonies was indicative of a "founder" event.

While the possibility of a single founder event in Hawaii is intriguing, resolution of this question by genetic techniques has thus far proven difficult. Since quantitative differences in cuticular hydrocarbons have been detected among geographically disparate populations of *C. formosanus* (Haverty et al., 1990a; Su and Haverty, 1991), we proposed to test whether resolution of quantitative differences in cuticular hydrocarbon mixtures of different colonies of *C. formosanus* within the same population or geographic area was possible. We report here the results of a study to characterize the variation of cuticular hydrocarbons of different colonies of *C. formosanus* from the same population (all from the island of Oahu). We report differences between castes, between colonies, and within the population over time to assess seasonality. This examination of the intrinsic variation in cuticular hydrocarbons will serve as the basis for developing optimal sampling regimes for further examination of variation within the species and assessment of potential founder events around the world.

METHODS AND MATERIALS

Collection of Termites. Samples of *C. formosanus* were collected monthly for 25 months (September 1990 through September 1992) from seven colonies from four separate locations on the island of Oahu, Hawaii. Four colonies were on the campus of the University of Hawaii: two adjacent to Gilmore Hall (Upper Gilmore and Lower Gilmore), and one each adjacent to Pope Hall and the former location of the Publication Building. One colony each was located in Kaneohe, on the Waipio Peninsula, and at the Poamoho Experiment Station of the University of Hawaii. The colony at the Poamoho Experiment Station is not a natural colony; it was derived from an incipient colony of unknown laboratory stock and was placed on the site in 1979 by Dr. Minoru Tamashiro [see Strong and Grace (1993) for details]. Samples were collected from each colony using the trapping technique developed by Tamashiro et al. (1973).

Each month termites from each colony were separated from wood, carton, soil, and other debris and one sample each of 200 workers, 50 soldiers, and up to 50 of nymphs or alates were placed in Petri dishes and dried in a desiccator with Drierite®. Termites were dried while alive, until they died, and then for an additional five days. We found that killing termites before drying them in ambient, laboratory air often resulted in decomposition of the termites. Concurrently, each month two voucher samples from each colony consisting of two or three individuals from each caste present were preserved in 80% ethanol; one sample was kept at the University of Hawaii and one kept at the Pacific

Southwest Research Station in Albany, California. About every three to four months, accumulated dried samples were shipped to California for characterization of the cuticular hydrocarbons. Dried samples were held at ambient temperature in Hawaii and California until they were extracted.

Hydrocarbons of C. formosanus. Detailed descriptions of extraction procedures for specific termite species have been previously published elsewhere (Blomquist et al., 1979; Howard et al., 1978, 1982, 1988; Haverty et al., 1988, 1990a,b). In our laboratory in California, dried termite samples were transferred to clean 20-ml vials and immersed in 10 ml of *n*-hexane for ten min. The resulting extract of cuticular lipids was pipetted through four cm of activated Biosil A in a Pasteur pipette minicolumn to isolate the hydrocarbon components. The hydrocarbon extract was then evaporated to dryness under nitrogen and redissolved in 60 μl of hexane.

Gas chromatography-mass spectrometry (GC-MS) analyses of the hydrocarbons were performed on a Hewlett-Packard 5890 gas chromatograph equipped with a capillary column (25 m × 0.2 mm ID, HP-1) and a Hewlett-Packard 5970B Mass Selective Detector. The system was interfaced with HP Chemstation software for data acquisition and analysis. Helium was the carrier gas, column flow was 2 ml/min, and the GC-MS was operated in the split mode with a ratio of 7 : 1. The GC was temperature programmed from 200° to 320°C at 3°C/min with a final isothermal hold of 16 min. Electron impact (EI) mass spectra were obtained at 70 eV, and scanning was done from 55 to 640 mass units every 1.0 second.

Quantification of individual hydrocarbon peaks was done by integrating the total ion chromatogram resulting from summation of mass ion abundances. Because of the large number of peaks representing co-eluting compounds and the necessity of checking these peaks for the presence of all compounds, all samples were analyzed and quantified this way. Past analyses using a flame ionization detector, rather than a mass selective detector, gave poor resolution of later-eluting peaks (see Haverty et al., 1990a) and important information may have been lost regarding high molecular weight hydrocarbons (C37 and higher).

n-Alkanes and methyl-branched alkanes were identified by their mass spectral fragmentation patterns (Blomquist et al., 1987; Nelson et al., 1980; Pomonis et al., 1980) and corroborated by calculating equivalent chain lengths (ECL) (Nelson and Sukkestad, 1970; Jackson and Blomquist, 1976). Mass spectra of dimethylalkanes were interpreted as described in Page et al. (1990b) and Nelson et al. (1980). Mass spectra of a trimethylalkane was interpreted as described by Page et al. (1990a).

In the text and tables, shorthand nomenclature has been used to identify individual hydrocarbons or mixtures of hydrocarbons. For example, heptacosane becomes *n*-C27; 3-methylheptacosane becomes 3-MeC27; 3,15-dimethyl-

heptacosane becomes 3,15-DimeC27, and 13,15,17-trimethylnonacosane becomes 13,15,17-TrimeC29. Hydrocarbons are presented in the table in the order of elution on our GC/MS system.

GC-MS peak areas were expressed as percentages of the total hydrocarbon fraction. The response variable for all statistical tests was the percentage of each individual peak, which may represent two or more co-eluting compounds (see Table 1). Summary statistics (mean and standard deviation of the percent of each peak or hydrocarbon component) were calculated for each caste combining all colonies and collection dates.

Intercaste, Intercolony, and Seasonal Variation in Hydrocarbons. We examined different transformations of the percentages of the hydrocarbons; none provided uniform improvement in skewness or kurtosis. As a result, all statistical treatments of data to assess intercaste, intercolony, and seasonal variation were conducted with actual percentages.

Differences between workers and soldiers were determined by a *t*-test of the percentage of each peak (or hydrocarbon component) with colonies (summed over all 25 collection periods) representing the replicates (i.e., $N = 7$ for each caste). Differences between colonies or months of the year were determined by analysis of variance of the percentage of each peak, separately for workers and soldiers. Statistical differences were tested to maintain an overall $\alpha = 0.05$ for the analysis of each of the 39 hydrocarbon peaks. Thus for each *t*-test or analysis of variance, the significance of the *t* or F statistic was tested at the $\alpha = 0.001$ level ($\alpha = 0.05/39$, where 39 is the number of statistical analyses conducted, one for each of the 39 hydrocarbon peaks).

Differences of hydrocarbon patterns between castes and between colonies were revealed by canonical discriminant analysis to provide two-dimensional displays of the chosen variables. Canonical discriminant analysis is a dimension-reduction technique related to principal component analysis and canonical correlation that derives a linear combination of quantitative variables (in this case, the percentage of hydrocarbon components) that summarizes between-class variation. The procedure that we used computes and tests Mahalanobis distances for pairwise comparisons of the colonies. Statistical analyses were accomplished with the TTEST, ANOVA, and CANDISC procedures of SAS System Release 6.08 (SAS 1990).

RESULTS AND DISCUSSION

Hydrocarbons of C. formosanus. Improvements in chromatographic equipment (replacing a flame ionization detector with a mass selective detector) and extraction technique (drying specimens before extraction with hexane and increasing the sample size to 200 individuals) allowed us to improve earlier

TABLE 1. HYDROCARBON MIXTURES (MEAN AND STANDARD DEVIATION) OF WORKERS, SOLDIERS, NYMPHS, AND ALATES OF *Coptotermes formosanus* FROM SEVEN COLONIES FROM OAHU, HAWAII, COLLECTED OVER 25 CONSECUTIVE MONTHS

Peak	Hydrocarbon[a]	Worker[d,e]	Soldier[d,e]	Nymph[d]	Alate[d]
1	*n*-C25	0.79(0.69)$^{\#}$	1.02(1.35)	0.55(0.48)	0.62(0.36)
2	9-; 11-; 13-MeC25[b]	2.19(0.67)*†	1.24(0.41)*†	1.38(0.50)	1.36(0.41)
3	2-MeC25 + 3-MeC25[c]	9.97(2.23)$^{\#}$	8.48(1.81)†	6.86(1.71)	8.55(1.85)
4	*n*-C26	0.86(0.65)$^{\#}$	1.20(0.38)$^{\#}$	0.79(0.51)	0.91(0.35)
5	9-; 10-; 11-; 12-; 13-MeC26[b]	2.71(0.73)*	1.77(0.39)*	2.11(0.69)	2.40(0.69)
6	2-MeC26 + 3-MeC26[c]	3.68(0.86)*$^{\#}$	2.95(0.62)*	3.16(1.03)	3.72(0.90)
7	*n*-C27	2.42(0.89)*$^{\#}$	5.66(2.07)*$^{\#\dagger}$	2.59(1.01)	3.23(0.65)
8	9-; 11-; 13-MeC27[b]	30.32(4.11)†	30.05(3.24)$^{\#}$	38.03(4.75)	37.54(6.26)
9	2-MeC27 + 9,13-DimeC27[c]	13.16(1.81)$^{\#\dagger}$	15.25(2.61)$^{\#}$	15.79(2.27)	16.16(1.26)
10	3-MeC27	3.53(0.67)*	6.17(0.90)*$^{\#}$	3.54(0.77)	4.69(0.94)
11	*n*-C28	0.45(0.54)$^{\#}$	0.63(0.33)$^{\#}$	0.43(0.31)	0.41(0.16)
12	3,15-; 3,17-DimeC27[b]	0.45(0.19)*$^{\#}$	0.07(0.13)*	0.30(0.16)	0.40(0.12)
13	9-; 10-; 11-; 12-; 13-; 14-MeC28 + 12,14-DimeC28[b,c]	3.84(0.59)*†	3.21(0.52)*	4.53(0.61)	4.36(0.83)
14	2-MeC28 + 9,13-DimeC28[c]	0.47(0.14)†	0.29(0.21)$^{\#}$	0.59(0.21)	0.52(0.18)
15	3-MeC28	0.14(0.09)*$^{\#}$	0.03(0.08)*$^{\#}$	0.19(0.11)	0.16(0.12)
16	*n*-C29	0.23(0.38)$^{\#}$	0.21(0.24)$^{\#}$	0.19(0.22)	0.13(0.09)
17	9-; 11-; 13-; 15-MeC29[b] + 13,15-DimeC29[c]	13.32(1.72)$^{\#}$	13.30(1.67)†	14.47(3.24)	12.19(1.89)
18	2-MeC29 + 9,13-DimeC29[c]	0.56(0.22)*	0.23(0.26)*$^{\#}$	0.49(0.21)	0.29(0.13)
19	3-MeC29	0.47(0.16)†	0.33(0.26)$^{\#}$	0.61(0.22)	0.48(0.19)
20	13,15,17-TrimeC29	1.02(0.28)$^{\#\dagger}$	0.85(0.35)	0.51(0.22)	0.50(0.14)
21	3,15-; 3,17-DimeC29 + 3,7-DimeC29[b]	0.24(0.10)*	0.01(0.03)*	0.16(0.10)	0.07(0.08)
22	14-; 15-MeC30[b] + 13,15-DimeC30[c]	0.37(0.11)*	0.15(0.18)*	0.33(0.15)	0.23(0.14)
23	13,15-; 15,17-DimeC31[b]	0.52(0.12)*†	0.27(0.18)*	0.28(0.14)	0.19(0.11)
24	11-; 13-; 15-; 17-MeC33[b]	0.20(0.10)*$^{\#}$	0.01(0.08)*	0.13(0.12)	0.02(0.03)
25	11-; 13-; 15-; 17-MeC35[b]	0.17(0.10)*	0.00(0.02)*	0.05(0.07)	0.00(0.00)
26	13-; 15-MeC37 + 15,17-DimeC37[c]	0.38(0.17)*$^{\#}$	0.11(0.16)*	0.04(0.06)	0.01(0.04)
27	13,17-; 15,19-DimeC37[b]	0.13(0.09)*†	0.01(0.06)*	0.00(0.00)	0.00(0.00)

TABLE 1. CONTINUED

Peak	Hydrocarbon[a]	Worker[d,e]	Soldier[d,e]	Nymph[d]	Alate[d]
28	14-; 15-; 16-MeC38[b]	0.09(0.09)*#	0.00(0.03)*	0.00(0.00)	0.00(0.00)
29	14,18-DimeC38	0.06(0.07)*#	0.00(0.03)*	0.00(0.00)	0.00(0.00)
30	13-; 15-MeC39[b] + 15,17-DimeC39[c]	0.96(0.45)#†	0.67(0.53)	0.16(0.12)	0.08(0.12)
31	13,17-; 15,19-DimeC39[b]	1.03(0.45)#†	0.66(0.55)#	0.17(0.12)	0.07(0.11)
32	13-; 14-; 15-; 16-; 17-; 18-MeC40[b]	0.23(0.18)†	0.12(0.21)	0.02(0.03)	0.00(0.00)
33	14,18-; 16,20-DimeC40[b]	0.22(0.22)*	0.08(0.24)*	0.02(0.03)	0.00(0.00)
34	13-;15-MeC41[b] + 15,17-DimeC41[c]	1.78(1.33)	1.90(1.33)	0.53(0.45)	0.25(0.23)
35	13,17-; 15,19-DimeC41[b]	1.82(1.27)	1.90(1.42)	0.60(1.01)	0.25(0.22)
36	15-; 16-; 17-MeC42[b]	0.22(0.30)	0.15(0.34)	0.05(0.05)	0.00(0.00)
37	14,18-DimeC42	0.09(0.20)	0.04(0.11)	0.02(0.03)	0.00(0.00)
38	13-; 15-MeC43[b] + 15,17-DimeC43[c]	0.70(0.81)	0.75(0.80)	0.25(0.18)	0.19(0.16)
39	13,17-DimeC43	0.22(0.36)	0.22(0.32)	0.06(0.07)	0.02(0.04)

[a] Carbon number is the total number of carbons in the parent chain, excluding methyl groups, e.g., 3-MeC25 = 3-methylpentacosane.
[b] An isomeric mixture. These components co-elute in this peak.
[c] Because of incomplete separation of these hydrocarbons both peaks are included as one value.
[d] Sample size = 175 for workers, 172 for soldiers, 42 for nymphs, and 9 for alates.
[e] Means (SD) for workers and soliders followed by "*" are significantly different at the $\alpha = 0.001$ level by t-test. Means followed by "#" represent significant differences among colonies for workers or soldiers, and means followed by "†" represent significant differences among collection months for workers or soldiers at the $\alpha = 0.001$ level by analysis of variance.

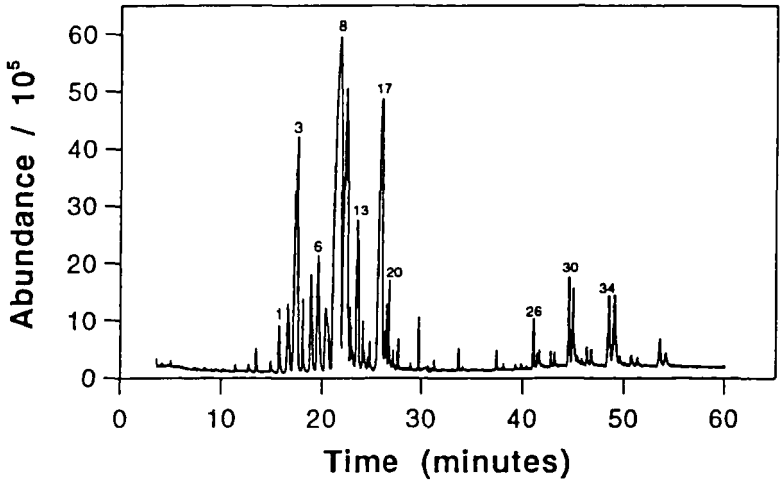

FIG. 1. Total ion chromatogram from 200 workers of *C. formosanus* from Pope Hall, University of Hawaii campus, Honolulu, Hawaii, in May 1992.

characterizations of the cuticular hydrocarbons of *C. formosanus* (Haverty et al., 1990a, 1991, 1992; McDaniel, 1990). We were able to resolve 39 individual peaks and characterize 52 individual or isomeric mixtures of hydrocarbons (Table 1 and Figure 1) from this species. Only *n*-alkanes and methyl-branched alkanes were found. *C. formosanus* does not make any olefins.

Normal alkanes present were *n*-C25, *n*-C26, *n*-C27, *n*-C28, and *n*-C29. Of the *n*-alkanes, *n*-C27 was the most abundant, comprising 2.4 and 5.7% of the total hydrocarbon for workers and soldiers, respectively, and intermediate amounts for nymphs and alates. The other *n*-alkanes generally represented no more than 1.2% each of the total hydrocarbons (Table 1).

Internally branched monomethylalkanes with methyl groups positioned at the nine carbon or greater elute about 0.7 carbon units in front of the *n*-alkane with the same number of total carbons. Their mass spectra have strong *m/z* ions representing cleavage internal to the methyl branch for which the ratio of even to odd is close to one (Blomquist et al., 1987). We identified isomeric mixtures of internally branched monomethylalkanes with parent carbon chains ranging from C25 to C43, except for C31, C32, C34, and C36. Positions of methyl branches ranged from carbon 9 to 18 (Table 1). Methyl branches located on even-numbered carbons were found only when the parent chain of the hydrocarbon had an even number of carbons, while branches on odd-numbered carbons were found to occur on hydrocarbons with either odd or even number of carbons in the parent chain. Internally branched monomethylalkanes were the

most abundant class of hydrocarbons produced by *C. formosanus*, representing roughly 45 to 50% of the total hydrocarbon. One isomeric mixture, 9-;11-;13-MeC27, accounted for over 30% of the total hydrocarbon for all castes surveyed (Table 1 and Figure 1).

2-Methylalkanes have an ECL of approximately 0.4 carbon units less than the corresponding *n*-alkane with the same number of total carbons and give a spectrum with a strong $(M-43)^+$ ion and a weaker $(M-15)^+$ ion. 3-Methylalkanes have an ECL of approximately 0.3 carbon units less than the corresponding *n*-alkane with the same number of total carbons and give a spectrum with a strong $(M-29)^+$ ion and a weaker $(M-57)^+$ ion. 2-Methyl- and 3-methylalkanes were identified for C25 to C29. In many cases the two peaks were not completely resolved on our system. These terminally branched monomethylalkanes comprise approximately 30% of the total hydrocarbons (Table 1 and Figure 1).

Internally branched dimethylalkanes constitute 15 to 20% of the total cuticular hydrocarbon fraction of *C. formosanus* (Table 1). There were two types of internally branched dimethylalkanes, those with either three methylene groups or those with one methylene group separating the methyl branches, representing roughly equal proportions of this class of compounds. Dimethylalkanes, such as 13,17-DimeC37 and 15,19-DimeC37, eluted about 1.4 carbon units before the corresponding *n*-alkane with the same number of total carbons, as did 14,18 and 16,20-dimethylalkanes. 9,13-Dimethylalkanes were present at C27, C28, and C29 and co-eluted with the 2-methylalkane of the same parent chain length.

Dimethylalkanes with one methylene group between methyl branches are not common in insects (Blomquist et al., 1987; Lockey, 1988), however, these compounds are fairly abundant in *C. formosanus* (Table 1). These alkanes have fragmentation patterns similar to other dimethylalkanes, except that an additional strong ion pair represents the daughter fragment formed by cleavage internal to the second methyl branch (Nelson et al., 1980). This ion pair has an even to odd ratio close to one. Another characteristic of this fragmentation is the relatively high intensity of the ion pairs representing the fragment ions formed by cleavage external to the second methyl branch, which have even to odd ratios of less than one (Nelson et al., 1980). The elution times for these compounds are 0.2 to 0.3 carbon units less than those of the other internally branched dimethylalkanes seen in this insect (Haverty et al., 1990a; Pomonis et al., 1980). Dimethylalkanes with one methylene spacing eluted slightly later than the monomethylalkane with the same parent chain length. Usually the two peaks did not fully resolve and thus were integrated as one peak. They occur together at C28, C29, C30, C37, C39, C41, and C43. The exception is the isomeric mixture 13,15-; 15,17-DimeC31, as no monomethyl alkane was identified at C31.

Terminally branched dimethylalkanes with the first branch on the 3-carbon had a strong $M-29^+$ ion and eluted about 1.0 carbon unit in front of the corresponding *n*-alkane with the same number of total carbons. These compounds

were not very abundant (less than 0.5 % of the total hydrocarbons) and occurred only at C27 and C29 (peaks 12 and 21, Table 1 and Figure 2). All were found to have the first methyl branch on the 3 carbon and the second on the 7, 15, or 17 carbon.

Only one trimethylalkane was identified: 13,15,17-TrimeC29. This compound was previously identified by McDaniel (1990). As in his study of *C. formosanus*, 13,15,17-TrimeC29 represented less than one percent of the total hydrocarbon. This is a symmetrical molecule with one methylene group separating each methyl branch, and it is not common in insects (Blomquist et al., 1987; Lockey, 1988). The ion patterns diagnostic for unusual, monomethylene interrupted spacing (1,1) were extrapolated from similar spectra for dimethylalkanes. The spectrum for 13,15,17-TrimeC29 is shown in Figure 2. The ion pair at 210/211 indicates a daughter fragment ion as described in the dimethyl section. The abundant ions at 238/239 and 280/281 represent fragment ions formed by cleavage external to the second and third methyl branches, respectively.

There were a few miscellaneous alkanes present in trace amounts that were inconsistently detected (i.e., 2-MeC24; 3,7-DimeC31) or that were in such small quantities as to be difficult to characterize and/or quantify. These compounds were disregarded for the quantitative purposes of this study.

Comparisons with Earlier Studies of Hydrocarbons of C. formosanus. The cuticular hydrocarbons of *C. formosanus* in the United States were previously characterized concurrently by Haverty et al. (1990a, 1992) and McDaniel (1990). McDaniel (1990) identified numerous mono- and dimethylalkanes, in trace amounts, in *C. formosanus* [see Table 1, McDaniel (1990)] that were not detected by Haverty et al. (1990a). McDaniel (1990) also identified the unique trimethylalkane, 13,15,17-TrimeC29, in trace amounts. In the study reported in this paper, we found and identified most of these compounds as well as numerous other hydrocarbons not seen by McDaniel (1990) or Haverty et al. (1990a, 1992). These include: *n*-C28, *n*-C29, 2-MeC28, 2-MeC29, 3-MeC28, 3-MeC29, 14-;15-MeC30, 14-;15-;16-MeC38, 13-;14-;15-;16-;17-;18-MeC40, 15-;16-;17-MeC42, 3,15-;3,17-DimeC27, 3,15-;3,17-DimeC29, 9,13-DimeC27, 9,13-DimeC28, 9,13-DimeC29, 12,14-;13,15-DimeC28, 13,15-DimeC29, 13,15-DimeC30, 13,15-;15,17-DimeC31, 15,17-DimeC37, 15,17-DimeC39, 15,17-DimeC41, 15,17-DimeC43, 14,18-DimeC38, 14,18-; 16,20-DimeC40, 14,18-DimeC42.

The possible reasons that these hydrocarbons were not detected in previous studies may be that sample sizes were inadequate [Haverty et al. (1990a) used only 50 to 100 workers or soldiers], or that the termites were processed when fresh or frozen rather than dried. Haverty et al. (1996) recently found that the extraction of hydrocarbons is more efficient if the specimens are dry rather than fresh or frozen. In our earliest investigations of hydrocarbons of *C. formosanus* (Haverty et al., 1990a), quantification was done using a flame ionization detec-

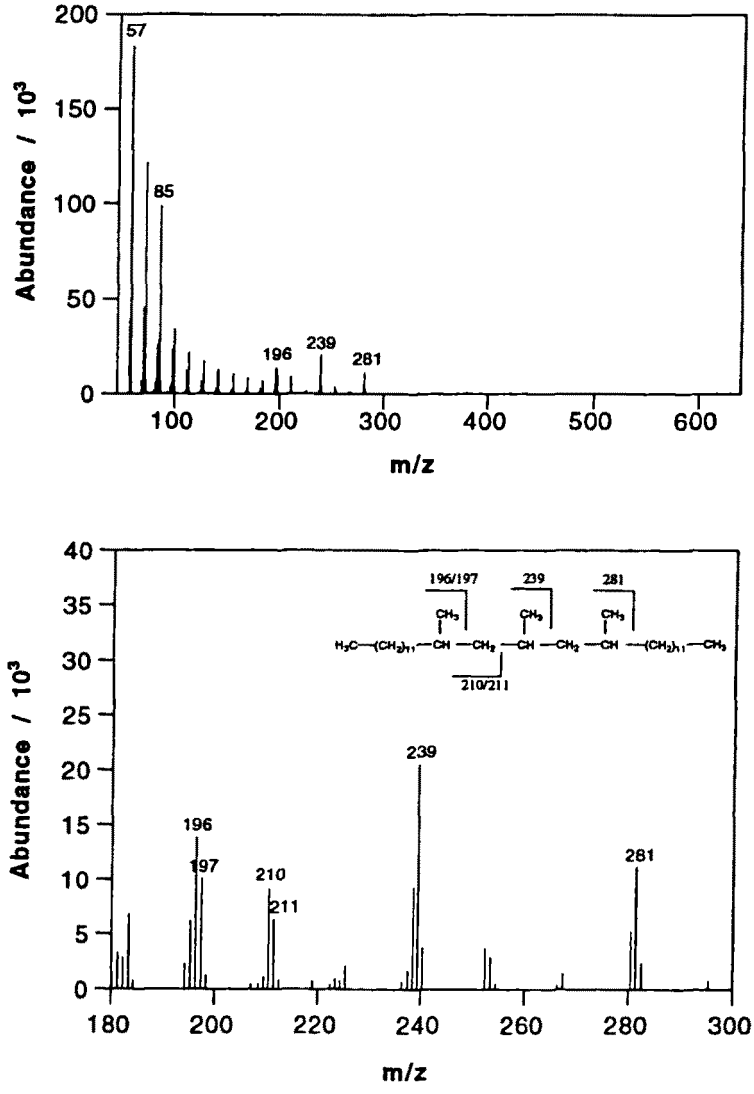

FIG. 2. Mass spectrum of 13,15,17-trimethylnonacosane from 200 workers of *C. formosanus* from Pope Hall, University of Hawaii campus, Honolulu, Hawaii, in September 1992.

tor; many of the smaller peaks, especially those at higher molecular weights (>C31), were not resolved. In addition, samples used for GS-MS analyses by Haverty et al. (1990a) were apparently not of sufficient concentration to detect and identify all of these peaks.

Intercaste Variation in Hydrocarbons. The cuticular hydrocarbon patterns of all four castes examined appear to be qualitatively quite similar. When we examined quantitative differences between workers and soldiers we found 19 of the 39 peaks to be statistically different at the $\alpha = 0.001$ level (Table 1). A few of these differences involve hydrocarbons that rarely were seen in the soldier samples, such as peaks 12, 15, 21, 24, 25, 27, 28, and 29 (Table 1). These hydrocarbons occur in workers in trace to slight amounts ($\leq 0.24\%$); in many worker samples these peaks were not resolved either. We suggest the absence of these peaks in soldier samples might be the result of a smaller sample size, resulting in less absolute hydrocarbon in the sample, i.e., the soldier samples were likely more dilute than those of the workers. Soldier samples included only 50 individuals whereas worker samples included 200 individuals. We did not, however, calculate differences in absolute quantities of hydrocarbons of any caste.

Nymphs and alates were collected on very few occasions compared to workers and soldiers. Because of the sporadic nature of these collections we did not compare them statistically to either workers or soldiers; nymphs and alates occurred seasonally and were not collected equally over the 25 collection periods. Many peaks in nymphs (27–29, 32, and 33) and alates (25–29, 31–33, 36, and 37) were seldom resolved and usually resulted in trace amounts. In some cases (peaks 27–29, 32–33, and 36–37) the hydrocarbons were never detected for alates and only occasionally for nymphs. These peaks that occur in trace amounts or are absent in nymphs and alates occurred in minute amounts ($<0.38\%$) in workers, with the exception of 13,17-; 15,19-DimeC39, which represented an average of 1.03% of the total hydrocarbon in workers. As was the case with soldiers, we attribute the absence of these peaks in nymph and alate samples to a smaller sample size, resulting in less absolute hydrocarbon in the sample. Nymph and alate samples included, at the most, 50 individuals whereas worker samples included 200 individuals.

Our sampling regime for this study was so extensive that the differences in hydrocarbon mixtures among the castes were easily displayed using canonical discriminant analysis (Figure 3). Soldiers and workers are statistically different from one another and from nymphs and alates at the $\alpha = 0.001$ level (Table 2). Nymphs and alates were not significantly different from one another at $\alpha = 0.001$.

Brown et al. (1996) found similar intercaste differences in *Drepanotermes perniger* (Froggatt) in Australia. All castes and stages were qualitatively similar

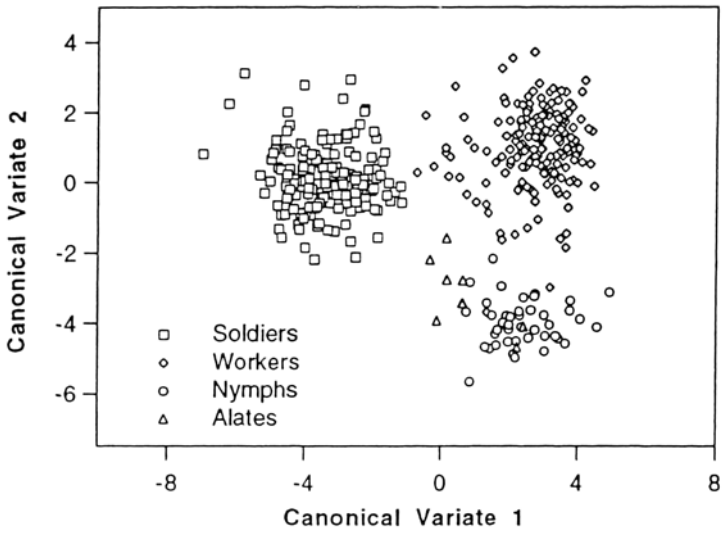

FIG. 3. Plot of four castes of *C. formosanus* from seven colonies from four locations in Oahu, Hawaii, along two axes of canonical discriminant space.

and quantitative differences were judged to be minor. The main differences were in amounts of 3-MeC27, I-MeC27, 3,X-DimeC25, and 3,X-DimeC27. Castes were easily separated by canonical variate analysis; the differences appear to diminish with age of the individuals.

Intercolony Variation in Hydrocarbons. We assessed differences between colonies separately for hydrocarbons of workers and soldiers by analysis of variance for each peak. We detected statistically significant differences (α = 0.001) between colonies in 18 peaks of workers and 12 peaks of soldiers. One of the goals of this research project was to separate or identify colonies on the

TABLE 2. MAHALANOBIS DISTANCE BETWEEN CASTES OF *Coptotermes formosanus* SHIRAKI FROM SEVEN COLONIES FROM FOUR LOCATIONS ON OAHU, HAWAII[a]

Caste	Worker	Nymph	Alate
Soldier	37.82†	50.07†	32.24†
Worker		25.11†	26.29†
Nymph			9.74

[a]Probabilities of Mahalanobis distance test the different between each value and zero. Distances with an '†' are statistically greater than zero at the α = 0.001 level.

basis of their hydrocarbon mixtures. Because there were so many statistically significant differences and they seldom involved the same hydrocarbons, we used all hydrocarbons in canonical discriminant analysis.

We were able to display differences between colonies with plots of hydrocarbon proportions along two axes of canonical discriminant space (Figure 4) and to test the similarity between colonies. Statistical analyses of Mahalanobis distances for all possible comparisons of workers or soldiers from these seven colonies indicate that all distances are statistically different from zero (Table 3). In other words, each of these seven colonies of *C. formosanus* can be separated from all the others on the basis of the concentrations of their cuticular hydrocarbon components.

Brown et al. (1996) used canonical variate analysis to separate geographic localities of *D. perniger*. Intercolony differences (from the same geographic location) were much smaller than geographic differences. As with intercaste differences, the absolute differences between geographic areas were small.

Seasonal Variation in Hydrocarbons. When we examined each of the 39 hydrocarbon values for the 25 collection periods, we obtained 30 statistically significant differences for workers and 24 for soldiers. Such a large number of statistically significant differences may simply be a result of differences between the extreme values (high and low) for the quantities of hydrocarbons over the 25-month period. It is quite possible that so many significant differences resulted from artifacts of technique. These problems with technique could involve slight differences in the way the termites were dried and/or held in our laboratories in Hawaii or California over the 25 months of this study. Also, the chromatographic sensitivity could very well have changed over that period of time. Both of these effects could have been operating concurrently. A more likely explanation is that there was, in fact, a seasonal effect.

To test the hypothesis of a seasonal effect, we combined our samples so that we were evaluating differences between months of the year rather than 25 separate, sequential collection periods. Thus, for each month of the year, we characterized the seven colonies twice, except for September; this month we characterized the seven colonies three times. In this way, we felt that we could determine any seasonal differences, not just chance statistically significant differences among the 25 collection periods, that were repeatable over the 2-year period.

We detected statistically significant differences ($\alpha = 0.001$) between months of the year for 12 hydrocarbon peaks of workers and 4 peaks from soldiers. We then visually examined the monthly trends of each of these statistically significant hydrocarbon peaks to determine whether the differences were due to a seasonal trend or simply due to extreme differences between the months with the most and least amount of the particular hydrocarbon peak. We found that two different hydrocarbon peaks each for workers and soldiers showed distinct

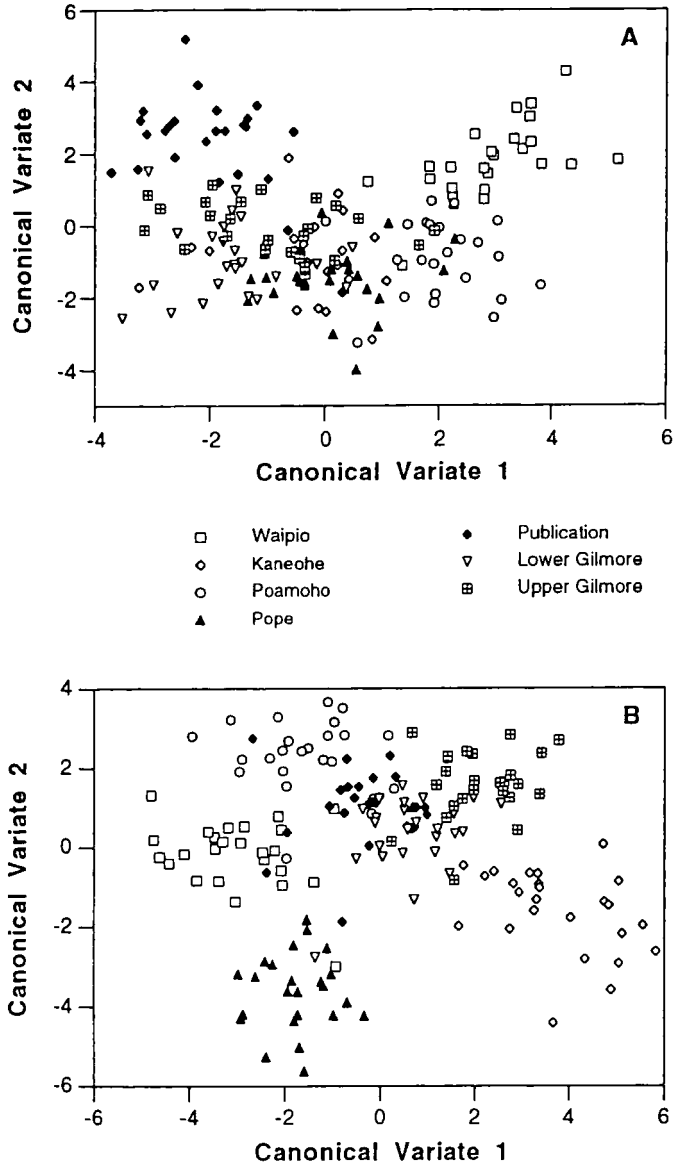

FIG. 4. Plot of seven colonies of *C. formosanus* from four locations in Oahu, Hawaii, along two axes of canonical discriminant space; A = workers, B = soldiers.

TABLE 3. MAHALANOBIS DISTANCE BETWEEN COLONIES, FOR BOTH WORKERS AND SOLDIERS, OF *Coptotermes formosanus* SHIRAKI FROM FOUR LOCATIONS ON OAHU, HAWAII[a]

Colony[b]	2	3	4	5	6	7
			Soldiers			
1	22.58	18.63	23.53	29.95	31.52	21.98
2		20.31	10.70	21.40	13.79	10.56
3			19.91	32.97	19.84	19.51
4				25.42	15.97	14.19
5					17.56	15.50
6						7.81†
			Workers			
1	54.91	25.93	26.64	33.78	24.50	36.73
2		47.09	41.19	38.39	25.52	27.47
3			38.86	23.08	23.06	30.77
4				33.64	25.18	45.57
5					18.28	31.15
6						10.96

[a]Probabilities of Mahalanobis distance test the difference between each value and zero. Distances with an '†' are **not** statistically greater than zero at the $\alpha = 0.001$ level.
[b]Colonies are as follows: 1 = Waipio, 2 = Kaneohe, 3 = Poamoho, 4 = Pope Hall, 5 = Publication City, 6 = Lower Gilmore, and 7 = Upper Gilmore.

seasonal trends (Figure 5). These trends were also obvious when colonies were examined separately for each of these hydrocarbon peaks. For these particular peaks, the trends were not an artifact of one or two colonies determining the average value for the month nor were they an artifact of technique. The differences were truly seasonal and were repeated from year to year. The statistically significant differences for the other peaks (10 for workers and 2 for soldiers) were due to differences between the extreme values; no seasonal trend was evident.

The biological significance of these trends is not obvious, but the major shifts in proportions for these hydrocarbon peaks correlate with the timing of the production of alates of *C. formosanus* on Oahu. Although alates of *C. formosanus* can be collected throughout the year, the major flights occur in May through July (Bess, 1970). This is almost exactly the same period (April through June) that alates were found in the colonies we sampled.

It seems reasonable to assume that these statistically significant shifts in amounts of hydrocarbon components are associated with the flight of the alates.

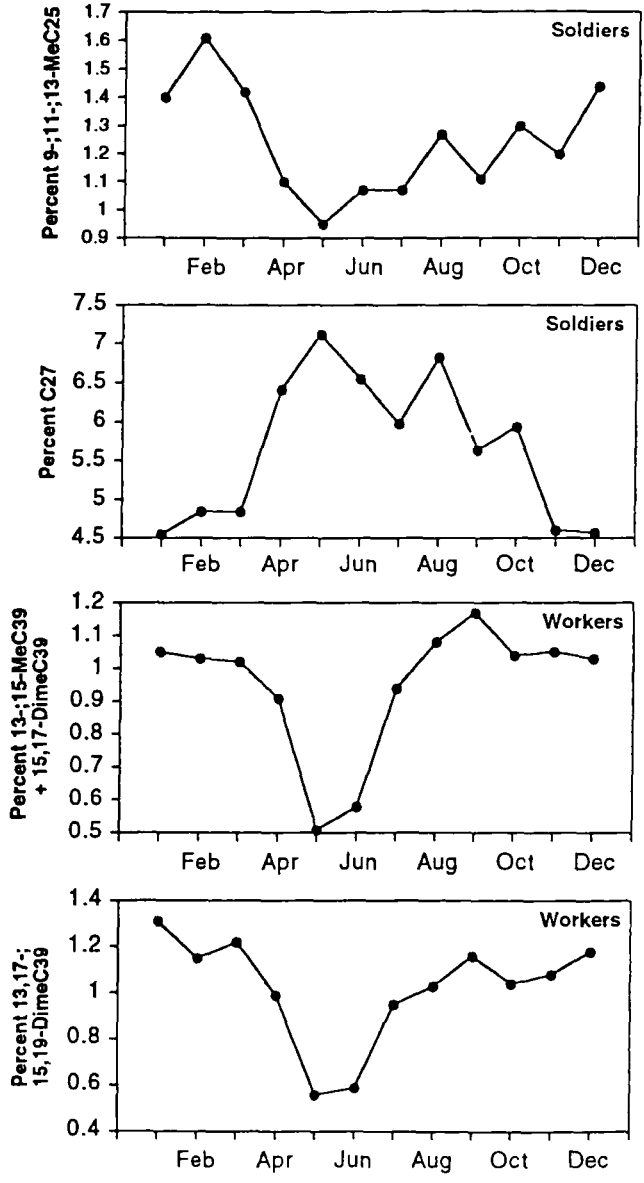

FIG. 5. Seasonal differences in hydrocarbons of *C. formosanus* soldiers (9-; 11-; 13-MeC25 and C27) and workers (13-; 15-MeC39 + 15,17-DimeC39 and 13,17-; 15,19-DimeC39) as a percentage of total hydrocarbons detected.

With the production of imagoes, the mature nymphs have metamorphosed and some individuals in the "worker" category will then differentiate to immature nymphs during this period. This would likely change the composition of the individuals remaining in the "worker" classification. Similarly, as the flight season approaches, termite colonies usually increase their investment in soldiers (Haverty and Howard, 1981; Howard and Haverty, 1981). Thus, a significant proportion of the individuals in the "worker" category are also differentiating to soldiers in April and May. After each flight many soldiers leave the nest to provide protection for the departing alates. Many of these soldiers are sealed out of the nest and lost to the colony (Bess, 1970; Nutting, 1969). The events surrounding the flight of the alates will result in a mix of young and old soldiers which could possess slightly different hydrocarbon mixtures.

Our survey of hydrocarbon mixtures of the same colonies for 25 months does not yet confirm that these colonies were derived from a single introduction to the Hawaiian Islands, or at least introductions of very closely related individuals. Given that *C. vastator* Light is suspected to have been introduced into Hawaii, and apparently was established at one time (Bess, 1970), it is quite possible that *C. formosanus* was introduced (and established) numerous times into Hawaii. The volume of movement of civilian and military materiel from the Far East through the Port of Honolulu and Pearl Harbor could easily have resulted in multiple introductions. However, confirmation of one or more founder events will require a broader sampling of *C. formosanus* populations within Hawaii, and comparison with populations from distant regions, such as Japan, Taiwan, and North America.

From the results reported here, we know that intercolony variation is greater than intracolony variation in cuticular hydrocarbon composition. We also know that we can characterize the colonies by quantifying hydrocarbons from either workers or soldiers; we need not collect data from both castes. Therefore, we propose that subsequent research on the biogeography of *C. formosanus* emphasize sampling hydrocarbons only from workers from as many separate colonies as possible from a given location, similar to the study of *D. perniger* by Brown et al. (1996). To minimize seasonal variation it is advisable to sample all colonies at times other than during the flight season. This latter criterion has the disadvantage of not providing imagoes for voucher specimens, but does provide a greater "window" for collecting.

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AGGREGATION PHEROMONES OF *Drosophila immigrans*, *D. phalerata*, AND *D. subobscura*

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Abstract—Aggregation pheromones of *Drosophila immigrans*, *D. phalerata* and *D. subobscura* were demonstrated by testing attraction of adult flies to hexane extracts of the flies in a windtunnel bioassay. Extracts of adult males of all species attracted conspecific males and females. However, *D. subobscura* flies were attracted only when the extract was applied on food. GC-MS analysis identified (Z)-11-octadecenyl acetate (cVA) in the extracts of adult male *D. immigrans* and *D. phalerata*. Both species were attracted to synthetic cVA. Male and female *D. subobscura* produced 5,9-pentacosadiene, 5-pentacosene, 2-methylhexacosene and 5,9-heptacosadiene, while only male *D. subobscura* produced (Z)-5-tricosene and minor amounts of cVA.

Key Words—Aggregation pheromones, (Z)-11-octadecenyl acetate, (Z)-5-tricosene, 5,9-pentacosadiene, windtunnel, Drosophilidae.

INTRODUCTION

Drosophila flies that breed in ephemeral habitats such as decomposing fruits and mushrooms often have aggregated distributions (Rosewell et al., 1990;

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Jaenike and James, 1991). Aggregative behavior in *Drosophila* flies is mediated by pheromones that can act in concert with odors of the habitat of the flies and indicate a suitable habitat for mating and oviposition (Bartelt et al., 1986; Schaner et al., 1987). An aggregated distribution may reduce interspecific competition and facilitate coexistence between species (Atkinson and Shorrocks, 1981; Ives, 1988), but should also be important for finding mates. *Drosophila* aggregation pheromones are produced by males and attract flies of both sexes (Bartelt and Jackson, 1984; Bartelt et al., 1985; Jaenike et al., 1992). The pheromones are transferred to females during mating and transferred further to an oviposition site by female flies (Bartelt et al., 1985). It has been suggested that these pheromones have functions in addition to aggregation behaviors, such as preventing matings of newly mated females and interactions between males (Zawistowski and Richmond, 1986).

Aggregation pheromones of *Drosophila* are generally volatile esters, ketones or unsaturated hydrocarbons (Bartelt et al., 1985, 1988; Moats et al., 1987). Several species produce the same compound; for instance, species of the *Drosophila melanogaster* group all have (*Z*)-11-octadecenyl acetate as an aggregation pheromone (Bartelt et al., 1985; Schaner et al., 1987, 1989). Such nonspecific pheromones can cause interspecific aggregates of related flies in a breeding habitat (Jaenike et al., 1992). However, flies also have a range of cuticular hydrocarbons that are assumed to take part in regulating mating behaviors (Scott et al., 1988; Cobb and Jallon, 1990). A mixture of these compounds may give species specificity at close range.

Apart from increasing the risk of intraspecific competition, aggregation pheromones can also affect the risk of parasitization as parasitoid wasps are attracted to the pheromones of adult *Drosophila* flies when searching for their larval hosts (Wiskerke et al., 1993). However, the risk of an individual *Drosophila* larvae to be attacked by a parasitoid should decrease at increasing densities of flies according to the functional response of the parasitoid (Hertlein and Thorarinsson, 1987). Recently a study on parasitoid wasps showed new and unexpected results when both generalist and specialist larval parasitoids of drosophilids were attracted to aggregation pheromones of adults of some host as well as some nonhost species (Hedlund et al., 1996). To interpret the results of the latter study, the aggregation pheromones of the *Drosophila* species involved need to be chemically identified.

From the study of parasitoid-host interactions (Hedlund et al., 1996) we have chosen three species, *Drosophila phalerata* (Meigen) and *Drosophila subobscura* (Collin) with a European distribution and one cosmopolitan species, *Drosophila immigrans* (Sturtevant) (Wheeler, 1981). This study aims at identifying aggregation pheromones of these three species not only to increase the knowledge of communication of *Drosophila* but also that of parasitoid-host interactions.

METHODS AND MATERIALS

Fly Cultures

Drosophila immigrans was reared on a medium of apple, water, agar, sugar and yeast. The culture originated from flies reared out of fruits collected in orchards in The Netherlands.

D. subobscura was reared in the same way as *D. immigrans*. The culture was established from flies reared from sap fluxes of trees originating from The Netherlands.

D. phalerata was reared on decaying *Agaricus hortensis* mushrooms. The culture originated from flies reared out of *Phallus impudicus* mushrooms collected in The Netherlands.

The flies were reared in 250 ml glass vials at 20°C on a 16:8 (L:D) photocycle. Fly pupae were washed out of the vial and transferred to 200 ml glass vials containing a layer of water agar.

Fly Extracts. Flies were separated by sex 0–6 hr after emergence from the pupae and transferred to new glass vials with a layer of water agar and honey on a strip of filter paper. To get sexually immature and mature flies the flies were collected at day one and day seven, respectively, and soaked in hexane (100 flies ml^{-1} hexane) at room temperature. The crude extracts were removed after 24 hr and kept at −20°C until further analysis.

GC-MS Analysis. The crude extracts were analyzed with gas chromatography (GC) and mass spectrometry (MS). Male- and female-derived extracts were compared by capillary gas chromatography (DB-1 column, 15 m length, 1 μm film thickness, 0.25 mm ID; temperature programmed from 50°C to 300°C at 10°C per min, with a final hold of 10 min at 300°C). GC retention indices relative to *n*-alkanes were calculated for sex-specific compounds and other, relatively abundant compounds. The Hewlett Packard 5890 gas chromatograph was equipped with splitless injector, flame ionization detector, and Hewlett Packard 3396A integrator. Electron impact mass spectra were obtained for the detected sex-specific compounds on a Hewlett Packard 5970 Mass Selective Detector with capillary GC inlet. Additional analyses were performed after removal of fatty acids by washing samples with 5% (w:w) Na_2CO_3 solution, since the large broad, fatty acid GC peaks could have obscured minor sex-specific compounds.

Saturated hydrocarbons were identified from mass spectra and GC retention indices. Unsaturated hydrocarbons and esters were identified by mass spectra, GC retentions, and mass spectra of dimethyldisulfide (DMDS) adducts (Carlson et al., 1989). The DMDS derivatives allowed location of double bonds, and double bond configuration was determined by GC retention in comparison with authentic standards. Sex-specific compounds were considered as possible pher-

omone components. When possible, synthetic versions of the sex-specific compounds were evaluated in the bioassay to verify behavioral activity.

Synthetic Compounds. (*Z*)-11-octadecenyl acetate, also called cis-vaccenyl acetate (cVA) was purchased from Sigma (St Louis, USA). The 5-tricosenes were prepared by the method of Sonnet (1974) from pentanal and (octadecyl)triphenylphosphonium bromide, and the *Z* and *E* isomers were separated by $AgNO_3$-HPLC as described by Heath and Sonnet (1980).

Bioassays. All bioassays were done in a cage (width 30 cm, depth 40 cm, height 35 cm) with nylon mesh (0.1 mm) along its long sides. A fan outside the cage generated an air flow of 0.1 m s^{-1} inside the cage. Each cage was stocked with 300 flies 16 hr before the tests. The flies were two to nine days old and of equal sex ratio. They were left with honey and water until the tests started. Fly extracts or synthetic compounds were applied to a filter paper (1 cm × 6 cm strip). The solvent was allowed to evaporate and the filter paper was inserted into a small glass vial (5.5 cm × 1.5 cm diameter). A drop of water was applied on the bottom of the vial. When a food substrate was present in the vial, either a 5 mm layer of an apple-yeast mixture (15:1) (w:w) or pieces of decaying mushrooms (*Agaricus hortensis*) were placed at the bottom of the vial. In each test two vials were placed inside the cage and perpendicularly to the air flow. One vial contained a hexane control and the other an extract or a synthetic compound dissolved in hexane. Tests with *D. phalerata* and *D. subobscura* lasted for 5 min while tests with *D. immigrans* lasted for 10 min. During this period of time the flies were allowed to enter a vial. At the end of the test the vials were capped with a cotton plug. The number and sex of the flies were determined. Subsequent tests separated by 3 to 5 min between the tests were done with the same cage. The cage was stocked with fresh flies each experimental day. All tests were performed at 20°C.

Each treatment (female extract, male extract etc.) was replicated 10 to 20 times and a series of treatments was carried out each experimental day (block design) so that any variation due to daily activities of the flies could be eliminated. A treatment was compared to its control with a Wilcoxon two sample test (Sokal and Rohlf, 1981) and a Kruskal-Wallis (Hollander and Wolfe, 1973) test compared the number of flies in traps of different treatments.

RESULTS

Hexane extracts of mature male and female *D. immigrans* were attractive to both sexes (52% males, 48% females) when responses were compared to a hexane control (Table 1). However, more than twice as many flies responded to male extracts as to female extracts. The GC-MS analysis revealed that extracts of mature males contained small amounts (30 ng/male fly) of (*Z*)-11-hexade-

TABLE 1. BIOASSAY ACTIVITY (MEAN NO OF FLIES CAPTURED IN VIALS) OF *Drosophila immigrans* AND *D. phalerata* IN A WINDTUNNEL BIOASSAY TO 7.5 μg SYNTHETIC cVA ((Z)-11-OCTADECENYL ACETATE) AND CRUDE FLY EXTRACTS (IN EQUIVALENTS OF TEN FLIES)

Treatment		Bioassay activity			
pheromone	control	pheromone mean[a] (sd)	control mean[a] (sd)	p[b]	N
D. immigrans					
female extract	hexane	1.10a (1.85)	0.10a (0.32)	0.05	10
male extract	hexane	2.80b (2.30)	0.10a (0.32)	0.001	10
cVA	hexane	1.15a (0.75)	0.20a (0.41)	0.001	20
cVA & apple yeast	hexane & apple yeast	5.33b (4.58)	1.14b (1.01)	0.001	20
D. phalerata					
female extract	hexane	1.15a (1.12)	0.68a (0.95)	n.s.	20
male extract	hexane	8.15b (3.44)	0.75a (1.07)	0.001	20
cVA	hexane	2.40a (1.23)	0.55a (0.76)	0.001	20
cVA & mushroom	hexane & mushroom	3.40a (2.50)	1.20a (1.44)	0.01	20

[a]Means followed by different letters are significantly different at the 0.05 level according to multi comparison tests on Kruskal-Wallis rank sums (Hollander and Wolfe, 1973).

[b]Differences between pheromone treatment and the control treatment according to a Wilcoxon two sample test (Sokal and Rohlf, 1981).

cenyl acetate and large amounts (750 ng/male) of (Z)-octadecenyl actetate, i.e., cVA (Tables 2, 3). Both sexes also produced 2-methyloctacosane and 2-methyltriacontane. When synthetic cVA was presented in the bioassay, flies were attracted to cVA independent of sex (50% males, 50% females) (Table 1). A combination of cVA and food (apple and yeast) increased the number of flies attracted to cVA by about five times compared to tests based on cVA only. However, the proportions of flies attracted to cVA vs. control were the same in both cases.

Bioassays of hexane extracts of *D. phalerata* showed that flies of both sexes (53% males, 47% females) were attracted to adult male extracts (Table 1). The same compounds were identified in these extracts as in those produced by *D. immigrans*, i.e., (Z)-11-hexadecenyl acetate and cVA (Tables 2, 3). *D. phalerata* flies were also attracted to cVA in the bioassay (Table 1). However, when cVA was tested in combination with decaying mushrooms the response of the flies did not increase. The number of *D. phalerata* flies attracted to extracts of males was about two to three times higher than when cVA was tested alone or in combination with mushroom.

D. subobscura flies of both sexes (52% males, 48% females) were attracted to hexane extracts of male flies, but only when the extracts were applied on

TABLE 2. COMPOUNDS IDENTIFIED IN EXTRACTS OF ONE AND SEVEN DAYS OLD FLIES OF *Drosophila immigrans* AND *Drosophila phalerata*

Compound	Producing sex	Amount (ng/fly)	
		7 days	1 day
D. immigrans			
(*Z*)-11-hexadecenyl acetate	male	30	—
(*Z*)-11-octadecenyl acetate	male	750	180
2-methyloctacosane	male & female	240	100
2-methyltriacontane	male & female	240	200
D. phalerata		30	—
(*Z*)-11-hexadecenyl acetate	male	30	—
(*Z*)-11-octadecenyl acetate	male	750	80
2-methyloctacosane	male & female	150	200
2-methyltriacontane	male & female	120	150

food (apple and yeast) (Table 4). Identification of compounds in the extracts showed that mature male flies produced about 30 ng cVA/fly and 120 ng (*Z*)-5-tricosene/fly (Tables 3, 5). Both sexes of *D. subobscura* produced large amounts of 5,9-pentacosadiene and minor amounts of 5-pentacosene, 2-methylhexacosane and 5,9-heptacosadiene. Male and female *D. subobscura* produced 2-methyloctacosane and 2-methyltriacontane as did the other two *Drosophila* species. The male specific compounds, cVA and (*Z*) or (*E*)-tricosene, did not

TABLE 3. THE KEY MS IONS (*M/Z*) OF UNDERIVATIZED AND DERIVATIZED FLY EXTRACTS OF THE IDENTIFIED COMPOUNDS. GC RETENTION INDEX IS RELATIVE TO A STANDARD WITH *n*-ALKANES

Compound	GC retention index	Key MS ions (*m/z*)	Key MS ions for DMDS derivatives (*m/z*)
(*Z*)-11-octadecenyl acetate	2166	310, 250	404, 145, 259
(*Z*)-11-hexadecenyl acetate	1974	282, 222	117, 259,
(*Z*)-5-tricosene	2291	322	416, 117, 299
(*Z*)-5-pentacosene	2492	350	444, 117, 327
5,9-pentacosadiene	2476	348	(mono-DMDS adduct) 442, 117, 325, 171, 271
5,9-heptacosadiene	2679	376	(mono-DMDS adduct) 470, 117, 353, 299
2-methylhexacosane	2665	380, 365, 337	
2-methyloctacosane	2866	408, 393, 365	
2-methyltriacontane	3066	436, 421, 393	

TABLE 4. BIOASSAY ACTIVITY (MEAN NO OF FLIES CAPTURED IN VIALS) OF *Drosophila subobscura* TO CRUDE FLY EXTRACTS (TEN FLY EQUIVALENTS) AND SYNTHETIC (*Z*) AND (*E*)-TRICOSENE AND cVA, (Z)-11-OCTADECENYL ACETATE

Treatment		Bioassay activity			
pheromone	control	pheromone mean[a] (sd)	control mean[a] (sd)	p[b]	N
male extract	hexane	0.40a (0.74)	0.40a (0.63)	n.s.	15
male extract & apple yeast	hexane & apple yeast	4.13b (2.17)	2.47b (1.88)	0.05	15
female extract	hexane	0.11a (0.32)	0.27a (0.46)	n.s.	18
female extract & apple yeast	hexane & apple yeast	4.38b (4.01)	4.62b (4.59)	n.s.	13
(*Z*)-tricosene (1.2 μg)	hexane	0.07a (0.26)	0.07a (0.26)	n.s.	15
(*E*)-tricosene (1.2 μg)	hexane	0.27a (0.59)	0.27a (0.59)	n.s.	15
cVA (3.0 μg)	hexane	0.13a (0.35)	0.40a (0.51)	n.s.	15

[a] Means followed by different letters are significantly different at the 0.05 level according to multi comparison tests on Kruskal-Wallis rank sums (Hollander and Wolfe, 1973).

[b] Differences between the pheromone treatment and the control treatment according to a Wilcoxon two sample test (Sokal and Rohlf, 1981).

attract flies in the windtunnel bioassay (Table 4). However, as these compounds were not tested in combination with food, further experiments are needed to reveal the attractivity of the compounds together with a food substrate.

DISCUSSION

The aim of this study was to identify the aggregation pheromones of the three *Drosophila* species involved. Both males of *D. immigrans* and males of

TABLE 5. COMPOUNDS IDENTIFIED IN HEXANE EXTRACTS OF SEVEN DAYS OLD *Drosophila subobscura*

Identified compounds	Producing sex	Amount (ng/fly)
(Z)-11-octadecenyl acetate (cVA)	male	30
(Z)-5-tricosene	male	120
5,9-pentacosadiene	male & female	1000
(Z)-5-pentacosene	male & female	200
2-methylhexacosane	male & female	90
5,9-heptacosadiene	male & female	120
2-methyloctacosane	male & female	250
2-methyltriacontane	male & female	25

D. phalerata produce (*Z*)-11-octadecenyl acetate (cVA) as a major pheromone component. In both species extracts of male flies as well as synthetic cVA attract male and female flies, and we conclude that cVA is an aggregation pheromone compound. However, male extracts of *D. immigrans* attract twice as many flies as pure cVA, and in the case of *D. phalerata* male extracts attract almost four times as many flies. The pattern of higher attractivity to extracts than to pure cVA has been shown for other cVA-producing species such as *D. melanogaster* (Bartelt et al., 1985), whereas other species, such as *D. simulans*, show no difference in attractivity between fly extracts and synthetic cVA (Schaner et al., 1987). A higher level of attractivity to fly extracts than to pure cVA, or to female extracts as in *D. immigrans*, may indicate that there are other compounds present in the extracts that are active in eliciting aggregative behaviors of the flies. Other compounds may be produced in small amounts by both sexes but have to act in concert with cVA to attract flies. (*Z*)-11-hexadecenyl acetate that was found as a male specific compound of *D. immigrans* and *D. phalerata* is produced in small amounts and may be a possible coattractant. Although further work is needed to test the activity of (*Z*)-hexadecenyl acetate and chemically identify other active compounds, cVA can be regarded as a major aggregation pheromone of *D. immigrans* and *D. phalerata*.

Aggregative behavior of *D. subobscura* was only observed towards male fly extracts in combination with food of the flies. To identify the main aggregation pheromone compound(s) of *D. subobscura* further work is needed with testing attractivity of flies to combinations of identified compounds and food, and fractionation of the extracts. However, the identified hydrocarbons reveal a system quite different from that of *D. immigrans* and *D. phalerata*. Rather it has similarities with the aggregation pheromones of the *D. virilis* subgroup in which a combination of hydrocarbons and male specific compounds elicits aggregative behaviors (Bartelt et al., 1986).

The presence of food odors seems to be necessary for *D. subobscura* to respond to a pheromone whereas it increases the attractivity of cVA for *D. immigrans*. Coattraction of flies to food odors and aggregation pheromones may be a general pattern among many *Drosophila* species. The attractivity of aggregation pheromones such as cVA have been shown to increase in combination with food substrates for several fly species of the *melanogaster* species group (Schaner et al., 1987; 1989). Other types of pheromone compounds, e.g., tiglates of flies of the *virilis* species group, can also be synergistic in combination with food odors (Bartelt et al., 1986).

cVA, the aggregation pheromone of *D. immigrans* and *D. phalerata*, is produced by a number of species within the subgenus *Drosophila* and the subgenus *Sophophora* (Bartelt et al., 1985; Schaner et al., 1987, 1989; Jaenike et al., 1992). The risk of increasing interspecific competition that follows upon the use of the same aggregation pheromone compound may be reduced in nature

by different usage of microhabitats. Of the three species studied here only two produce cVA as the major pheromone compound and of these *D. immigrans* breeds mainly in fermenting fruit (Atkinson and Shorrocks, 1977), whereas *D. phalerata* is a woodland species that breeds in decaying mushrooms (Janssen et al., 1988). The attraction of flies to a combination of food odors and aggregation pheromones, as discussed above, will probably decrease the risk of competition between the species. However, fly species with similar aggregation pheromones and breeding sites have been shown to form interspecific aggregations (Jaenike et al., 1992).

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EXPERIMENTS ON BIOASSAY SENSITIVITY IN THE STUDY OF ALLELOPATHY

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Abstract—The purpose of these experiments was to study the effects of various experimental procedures and conditions on bioassay sensitivity in allelopathic studies. The following factors were considered: bioassay methods, choice of test species, the effect of osmotic potential on germination and growth of the test species, growth in dark or in light and the effect of concentrating the extracts in vacuo. Extracts from rape and rye were used in the studies to act as allelopathic agents. Ryegrass appeared as the most sensitive test species when grown horizontally on quartz sand, while radish was the most sensitive test species when grown on filter paper in transparent boxes at a 45° angle. Root length was the most sensitive growth variable measured. Percent germination appeared a more sensitive measure than the speed of germination index, "S", when germinated seeds were counted after one and two days for radish and ryegrass, respectively. A rise in the osmotic potential affected germination more than root length, and radish appeared more sensitive to a rise in osmotic potential than ryegrass. A confounding of germination and root length inhibition may therefore, give misleading results.

Key Words—Allelopathy, bioassay, evaporator, germination, growth conditions, growth parameters, osmotic potential, radicle elongation, sensitivity, test species.

INTRODUCTION

Bioassays are useful and necessary tools for studying the allelopathic potential of plant or soil extracts and for evaluating the activity of the extracts during purification and identification of allelopathic compounds. Nearly all reports on

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allelopathy describe some type of bioassay method used to demonstrate allelopathic activity (Leather and Einhellig, 1986). Generally there are two types of measurements for testing biological activity of allelopathic compounds: the measurements of specific biological activity (e.g., inhibition of photosynthesis) or measurements of some aspects of growth (e.g., germination, root dry weight). Putnam and Duke (1978) and Leather and Einhellig (1986) reviewed various bioassays for the study of allelopathy. The most widely used bioassay to test allelopathic activity in an extract is the technique of seed germination in petri dishes on filter paper, sand, soil or agar. Numerous studies also include plant growth bioassays, but the effect on germination is often not separated from the effect on growth (e.g., Liu and Lovett, 1993; Kato-Noguchi et al., 1994).

Various factors influence the sensitivity of a bioassay. Different test species may have varying sensitivity to specific allelopathic compounds (e.g. Krogmeier and Bremner, 1989) or to aqueous extracts (e.g., White et al., 1989). In some studies the main goal is to rank different species in their sensitivity to the active compounds (e.g., Smith and Martin, 1994), but in other studies it is important to select the most sensitive test species for detecting low limits of the allelopathic compounds. In both types of studies it is important to realize the possibility of an interaction between the test species and the bioassay method. Recording germination is a commonly used measurement, but several investigations have revealed that this is not the most sensitive parameter (Leather and Einhellig, 1986). Root length has often shown to be a more sensitive parameter, but roots are not as easy to measure as germination, and the design of the bioassay method may influence the growth of the roots. Water potential is important in the germination process (Bradbeer, 1988) but will also influence the growth of the seedlings. The osmotic potential will influence the results of the bioassay test, and variation in sensitivity to osmotic potential between test species may occur. Although Anderson and Loucks (1966) demonstrated the importance of solution osmotic potential when testing plant extracts, very few studies on allelopathic effects include osmotic effect (Leather and Einhellig, 1986). Wardle et al. (1992) found that the allelopathic effects could be substantially overestimated when the osmotic effects were not taken into account. Interactions between experimental conditions can also affect the results, as found between osmotic potential and water uptake in light or in dark for lettuce (*Lactuca sativa* L.) (Nabors and Lang, 1971).

Clearly a variety of bioassay methods and experimental factors influence the results of bioassays in the study of allelopathy. The lack of standardized bioassays makes comparisons between different studies very difficult, and even reports originating from the same laboratory differ in the conduct of the assay (Leather and Einhellig, 1986). Therefore, the aim of the present investigation was to study if, and in case to what extent, bioassay method, test species,

osmotic potential, light conditions, and evaporator treatments influence the results and the sensitivity of growth parameters in a bioassay.

METHODS AND MATERIALS

General

Winter-rape (*Brassica napur* L. ssp. *oleifera* DC. cv. "Bambu") and winter-rye (*Secale cereale* L. cv. "Danko") were greenhouse-grown in pots under day/night regime of 16/8 hr (High-pressure sodium lamps/15000 lux) and a temperature of 20°C. Above ground plant tissue from 40 days old plants of rape and rye were collected and cut separately into pieces of 2 to 3 cm length. Half of the plant matter was oven dried at 50°C for 72 hr before extraction. In the dry matter extraction a concentration of 1 g dry matter per 20 ml distilled water was prepared. As the fresh plant material was too voluminous, it was not possible to obtain the same concentration in fresh matter extraction. In this procedure, concentrations of 0.52 g dry matter of rye per 20 ml and 0.68 g dry matter of rape per 20 ml were made. The mixture of plant matter and distilled water was agitated for 5 hr on an orbital shaker at room temperature (24°C). Extracts were filtered through two metal-filters, 0.630 mm and 0.212 mm, before vacuum-filtration through filter paper (Whatman #1). To obtain the same concentration in the fresh matter extracts as in the dry matter extracts (1 g/20 ml on a dry matter basis), the fresh-matter extracts were reduced about 50% in vacuo (40°C), and then diluted to give concentrations of 1 g dry matter/20 ml. The extracts were stored at −20°C until used.

Radish (*Raphanus sativus* L., cv "Cherry Belle orig.") and Italian ryegrass (*Lolium multiforum* Lam. var. *italicum* cv "Mondora") were used as test species in the germination and growth bioassays. A 2 mm radicle length was considered as fulfilled germination. When used in growth assay, the seedlings were pregerminated for 48 hr.

Two types of bioassay methods were used in the experiments. Method 1 included plastic dishes (18 cm in diameter, 4 cm deep) containing 150 g of quartz-sand (mean particle size = 0.28 mm) covered with a glass plate. The quartz sand was moistened with 45 ml extract or distilled water (control). The seeds or seedlings were placed on the surface of the sand. As the accuracy of measurements of radicle elongation in petri dishes is questionable (Leather and Einhellig, 1988), a second bioassay method (Method 2) was developed to obtain easy and fast measurements of root length. For this method transparent square boxes (24 cm long, 11 cm wide and 7 cm high) were used with two layers of filter paper (Munktell 1701, art. no. 261 024) in the bottom of the box. The filter papers were moistened with 30 ml extract or distilled water (control). The

seedlings of the test species were placed on the filter paper along a horizontal line in the upper part of the box with the root pointed downwards. The boxes were arranged at a 45° angle which gave straight roots and root length was easy to measure. The transparent box-system was also used in the germination assay. It was then placed horizontally with two layers of filter paper in the bottom supplied with 20 ml extract or distilled water. All experiments were conducted under the same conditions, in a constantly illuminated (Luma Standard LE 58W Vit-S) growth chamber at 22°C.

Screening Experiment with Test Species and Plant Extracts Using Bioassay Method 1

The experiment was established as a screening experiment with the experimental factors of test species (radish and ryegrass), plant extracts (rape and rye), extracts from fresh or dried material, and two concentrations (1 g/20 ml and 1 g/60 ml). The dry and fresh matter extracts of rape and rye (1 g/20 ml) were diluted to give the concentration 1 g/60 ml. Bioassay Method 1 was used in this experiment. Fifty seeds of Italian ryegrass or radish were placed on the surface of the sand. Germinated seeds were counted after five days for radish and six days for ryegrass. Root length, shoot length, root dry weight, and shoot dry weight were measured on the seeds that germinated. A 2^k factorial design was used with $k = 4$ (Montgomery, 1984).

Experiment with Bioassay Methods and Test Species

This experiment was set up to separate the effects of germination and growth inhibition, to compare the two bioassay methods, and to study the interaction between bioassay methods and test species. The experiment covered the following concentrations: 0 (distilled water), 1 g/120 ml and 1 g/60 ml. Only dry matter extract of rape was used. To study the effect on germination, the extracts (20 ml each) were added to the transparent square boxes with filter paper. One hundred seeds of radish or ryegrass were placed in each box. Germinated seeds were counted and removed daily for five days. Both bioassay methods were used in the study of seedling growth with 20 seedlings of radish or ryegrass in each box/dish. Root length, shoot length, root dry weight, and shoot dry weight were measured after four days for both test species.

Experiment on the Effect of Osmotic Potential on Germination and Growth

The experiment was conducted to evaluate the effects of osmotic potential on germination and growth of radish and ryegrass. Five concentrations of polyethylene glycol 6000 MW (PEG) were evaluated: 0%, 5%, 10%, 15%, and 20% on a weight basis. Effects on germination were evaluated using horizontally

placed boxes and germinated seeds were counted and removed daily for five days. Bioassay Method 2 was used to evaluate the effects on root and shoot growth. Root and shoot length were measured after four days.

Osmotic potentials for dry matter extracts of rape and rye and PEG solutions were determined using a vapor-pressure osmometer. The potentials were the same for the rye and rape extracts and were for the 1/120, 1/60, and 1/20-concentrations 24, 54, and 157 mOsm, respectively. The 5, 10, and 15% PEG-solutions were selected to fit these figures and showed potentials of 17, 65, and 133 mOsm, respectively, while the 20% PEG concentration showed an osmotic potential of 252 mOsm.

Experiment on the Effect of Light on Root Length Inhibition

To study whether light conditions could affect radish root length sensitivity to allelopathic compounds, radish seedlings were grown in light or dark with distilled water or dry matter extract of rape (1 g/120 ml). Dark growth conditions were obtained by covering the transparent boxes with aluminum foil. Root length was measured after four days.

Experiment on the Effect of Evaporator Treatment

This experiment was conducted to evaluate whether an evaporator could have any deviating effect beyond the effect of concentrating the extracts. The evaporation procedure was as follows: the volume of the sample was reduced 50% in vacuo (40°C) followed by diluting the concentrated extracts with distilled water to obtain the starting concentrations of 1 g/120 ml and 1 g/60 ml, respectively. Dry matter extracts of rape and rye were used with radish seedlings as test species in bioassay Method 2. Root length was measured after four days.

Statistical Considerations

Speed of germination index "S" was calculated as described by Bradbeer (1988) and Wardle et al. (1991) as

$$S = \left[\frac{N_1}{1} + \frac{N_2}{2} + \frac{N_3}{3} + \frac{N_4}{4} + \frac{N_5}{5} \right] \times 100$$

Where N_1, N_2, . . . , N_5 are the proportion of seeds that germinated on day 1, 2 . . . 5. Except for percent germination and speed of germination, all variables were transformed to logarithms to homogenize variances and to allow proportional comparisons to be made. To evaluate the sensitivity of test species by using the actual values of root and shoot measurements (and not relative values), the interactions including the test species factor and the concentration factor can be used. When plotting logarithmic transformed values, the lines which can be

drawn for each species between concentrations would be parallel if there is no difference in sensitivity between the test species. If there are differences in sensitivity between test species, these lines would not be parallel, which would be shown by a significant interaction term of test species × concentrations. The advantage of using this technique for testing sensitivity in comparison with relative values (percent of control), is the avoidance of the less statistically appropriate relative values.

The experiments were established as complete factorial designs and analysis of variance was performed. Interactions including up to three factors were used in the statistical models, except for interactions with the replicate effect, which were all included in the error term. Logarithmic transformed values are presented as geometric means. All experiments were repeated three times, each time representing one replicate in the statistical analysis.

RESULTS

Screening Experiment with Test Species and Plant Extracts Using Bioassay Method 1

Radish was the more sensitive test species for all parameters observed (Table 1). Root length was the most sensitive parameter. Percent germination was about as sensitive as the measurements on shoots, but much less sensitive than root length. There were significant differences between effects of extracts from rape and rye on root length, root dry matter, and shoot dry matter, with the extract from rye giving the greatest reduction in growth of the test species. Although the effect was significant, the difference in root length between extracts from rape and rye was only 1 mm. Extract from a dried material was much more effective in reducing plant growth than those made of fresh material. As expected, phytotoxicity was more severe at the higher concentration than the lower one.

Experiment with Bioassay Methods and Test Species

Significant differences in cumulative germination between days, extract concentrations, and test species were recorded (Figure 1). The results for test species are presented in separate figures for convenience. There were greater differences between the concentrations during the first two days than later on, especially with ryegrass as test species. Sensitivity of the germination parameter was highest for radish at day one and at day two for ryegrass. The figures indicate a delay in germination for both species for both extract concentrations, which was also shown significantly by the speed of germination index "S" (Table 2). Radish germinated faster than ryegrass, while ryegrass germination seemed to be less affected by increasing extract concentration.

TABLE 1. MAIN EFFECTS OF TEST SPECIES, TYPE OF EXTRACT, AND CONCENTRATIONS ON GERMINATION, ROOT LENGTH AND DRY MATTER (DM) AND SHOOT LENGTH AND DM. VALUES WITHIN MAIN EFFECTS AND MEASUREMENT ARE CONSIDERED SIGNIFICANTLY DIFFERENT AT THE 5% LEVEL WHEN FOLLOWED BY DIFFERENT LETTERS (PERCENT OF CONTROL IN PARENTHESES)

		Root		Shoot	
	Germination % (% of control)	length mm (%)	DM mg (%)	length mm (%)	DM mg (%)
Test species:					
ryegrass	69a (79)	8a (41)	16a (58)	21a (76)	21a (76)
radish	43b (47)	2b (17)	19a (31)	2b (42)	91b (48)
Extract from:					
rape	57a (64)	5a (32)	19a (49)	7a (62)	49a (65)
rye	55a (62)	4b (26)	15b (42)	6a (56)	39b (59)
Material:					
fresh	74a (83)	14a (49)	37a (72)	14a (90)	83a (93)
dried	38b (43)	1b (10)	7b (17)	3b (29)	22b (32)
Concentration:					
1 g/60 ml	69a (78)	9a (41)	25a (59)	10a (76)	64a (76)
1 g/20 ml	43b (48)	2b (18)	11b (31)	4b (43)	29b (49)

Root and shoot length and shoot DM were more sensitive parameters in bioassay Method 2 than in Method 1 (Table 3). In this experiment, ryegrass was the more sensitive test species, while root length was the most sensitive parameter. The variance analysis showed, however, significant interaction in root length between test species, extract concentration and bioassay method ($P = 0.0001$), indicating that length of radish roots was most sensitive when using bioassay Method 2, while length of ryegrass roots was most sensitive when grown in Method 1.

The Effect of Osmotic Potential on Germination and Growth

The effects of PEG on germination are shown in Figure 2. All main factors and interactions appeared significant in the statistical analysis. Increasing proportion of PEG reduced the germination of ryegrass, and a considerable decrease in germination was recorded when PEG-concentration exceeded 15%. In radish this "threshold value" appeared already above 10% PEG. In the speed of germination index "S" (Table 4), radish had the fastest germination at 0 and 5% PEG, while ryegrass was the faster one at 10% PEG and above. In root length ryegrass was the least sensitive at 5, 10, and 15% PEG, while radish appeared less sensitive to changed osmotic potential than ryegrass at 20% (Table

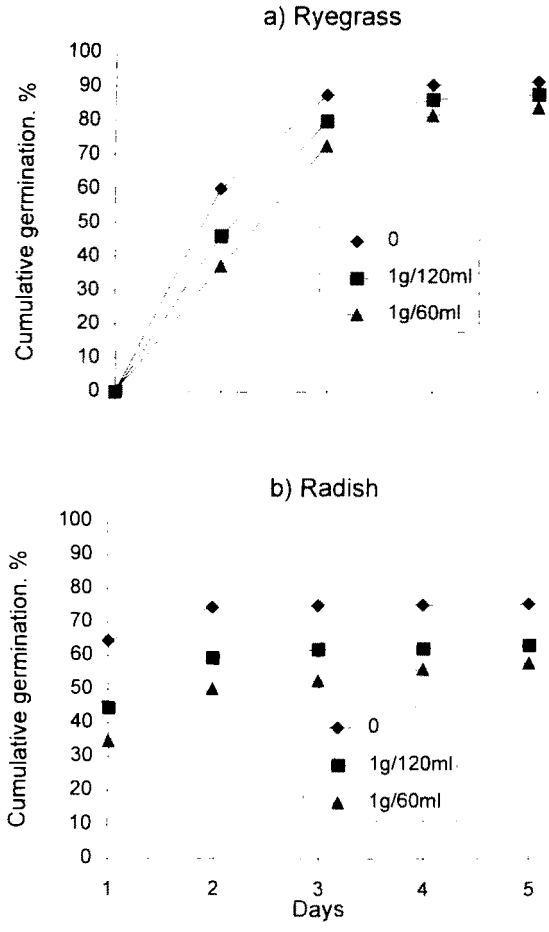

FIG. 1. The interaction between day of recording, plant extract concentration and the test species ryegrass (a) and radish (b) in cumulative germination ($p_{days \times test species \times concentration} = 0.01$).

5). Germination was more affected by increased osmotic potential than root length.

The Effect of Light on Root Length Inhibition

Radish root length was significantly longer when grown in dark than in light ($P = 0.0001$). There was also a significant interaction between the light

TABLE 2. THE INTERACTION BETWEEN TEST SPECIES AND EXTRACT CONCENTRATION IN SPEED OF GERMINATION INDEX 'S' (PERCENT OF CONTROL IN PARENTHESES)[a]

	Ryegrass	Radish
Concentration:		
0	40(100)	70 (100)
1 g/120 ml	36 (90)	53 (76)
1 g/60 ml	33 (83)	44 (63)

[a] $P_{\text{test species} \times \text{concentration}} = 0.0003$

factor and extract concentration ($P = 0.006$), showing that radish root length was a more sensitive parameter when grown in dark than in light.

The Effect of Evaporator Treatment

The phytotoxicity of the extracts was not changed during evaporation. Neither could a significant interaction between the evaporation factor and the extracts be shown.

TABLE 3. MAIN EFFECTS OF TEST SPECIES, TYPE OF BIOASSAY METHOD, AND CONCENTRATIONS ON ROOT LENGTH AND DRY MATTER (DM) AND SHOOT LENGTH AND DM. VALUES WITHIN MAIN EFFECTS AND MEASUREMENT ARE CONSIDERED SIGNIFICANTLY DIFFERENT AT THE 5%-LEVEL WHEN FOLLOWED BY DIFFERENT LETTERS (PERCENT OF CONTROL IN PARENTHESES)

	Root		Shoot	
	length mm (%)	DM mg (%)	length mm (%)	DM mg (%)
Test species:				
ryegrass	36a (62)	20a (89)	76a (114)	32a (114)
radish	29b (69)	40b (109)	15b (141)	130b (149)
Bioassay:				
Method 1	27a (68)	26a (85)	38a (141)	62a (150)
Method 2	38b (63)	31a (112)	29b (115)	67a (113)
Concentration:				
0	56a (100)	31a (100)	27a (100)	54a (100)
1 g/120 ml	28b (53)	30a (107)	37b (141)	70b (149)
1 g/60 ml	21c (42)	24a (89)	37b (142)	71b (146)

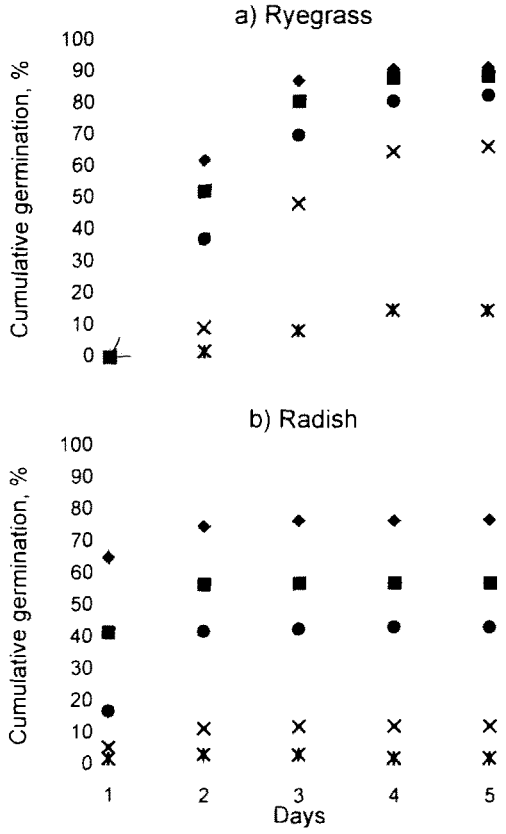

FIG. 2. The interaction between day of recording, polyethylene glycol 6000 MW (PEG)-concentration and the test species ryegrass (a) and radish (b) in cumulative germination. PEG concentrations are 0% (-◆-) 5% (-■-), 10% (-●-), 15% (-×-) and 20% (-*-) on a weight basis ($p_{\text{days} \times \text{PEG-concentration} \times \text{testspecies}} = 0.0001$).

DISCUSSION

Percent germination has commonly been used to measure the effects of allelopathic compounds. This is a rapid method for a large number of samples. However, Leather and Einhellig (1985) indicated that percent germination may not be the most sensitive parameter. Speed of germination may be a more sensitive indicator of allelopathic effects (Wardle et al., 1991) as allelochemicals may delay germination rather than affect the final germination percentage. In the present investigation percent germination was a more sensitive indicator than

TABLE 4. THE INTERACTION BETWEEN TEST SPECIES AND POLYETHYLENE GLYCOL 6000 MW (PEG)-CONCENTRATION IN SPEED OF GERMINATION INDEX "S"[a]

	S (% of control)	
	ryegrass	radish
PEG-concentration:		
0	41 (100)	71 (100)
5	38 (93)	50 (70)
10	33 (80)	30 (42)
15	22 (54)	9 (13)
20	5 (12)	2 (3)

[a] $p_{\text{test species} \times \text{PEG-concentration}} = 0.0001$

speed of germination, one and two days after initiating the germination bioassay for radish and ryegrass, respectively. In addition to being more sensitive, recording data one or two days after sowing is less time consuming than daily recordings for several days required for computing the speed of germination index. In the present investigation, root length was the most sensitive parameter for detecting growth retarding substances. These results are consistent with previous studies (e.g., Leather and Einhellig, 1986; Wardle et al., 1991; Wardle et al., 1992).

The screening experiment, in which the effect on germination and growth was confounded, indicated that radish was a more sensitive test species than ryegrass. The experiment which included both bioassay methods on the other hand showed that test species sensitivity depends on bioassay method, and rye-

TABLE 5. THE INTERACTION BETWEEN TEST SPECIES AND POLYETHYLENE GLYCOL 6000 MW (PEG)-CONCENTRATIONS IN ROOT AND SHOOT LENGTH

	Root mm (% of control)		Shoot mm (% of control)	
	Ryegrass	Radish	Ryegrass	Radish
PEG-concentration:				
0	71 (100)	83 (100)	66 (100)	10 (100)
5	72 (102)	73 (89)	64 (97)	9 (87)
10	70 (98)	61 (74)	51 (78)	8 (74)
15	53 (75)	59 (72)	21 (32)	7 (65)
20	20 (30)	36 (44)	3 (5)	6 (65)
P-values	0.01		0.0001	

grass was the more sensitive species when bioassay Method 1 was used, while radish was the more sensitive when Method 2 was used. These contradicting results may be explained by the confounding of germination and growth inhibition in the screening experiment. Germination of radish was more affected by the extracts than germination of ryegrass. Also, when evaluating the effects of osmotic potential on germination by using PEG, we found that radish was more sensitive to a rise in osmotic potential than ryegrass. The PEG experiment was conducted in continuous light, and germination of radish can be inhibited by light when exposed to osmotica (Bewley and Black, 1994), while no inhibition occurs when germinated in darkness. This kind of interaction may also have caused the differences in sensitivity between the two test species.

Comparison of PEG-solutions and extracts with similar osmolality, showed that the effect of PEG was much lower than those of the extracts indicating that the activity of the extracts is not an effect of osmotic potential alone. Osmotic potential also affected germination more than root length. This shows, as the contradicting results of the experiments with plant extracts also do, the importance of separating effects on germination from effects on growth.

Radish root length was a more sensitive variable in dark than in light. Other species react to light conditions in other ways. Shilling and Yoshikawa (1987) found that root growth of barnyardgrass (*Echinochloa crus-galli* (L.) PB.) and hemp sesbania (*Sesbania exaltata* (Raf.) Cory) were more sensitive under light conditions, and that all measurements of shoot growth were generally more inhibited in the dark than under light conditions. The species to be used in bioassays should therefore, be considered under which circumstances it is most sensitive.

We have compared two types of bioassays. When the test species were grown in boxes at 45° on filter paper, the root growth was straight and much easier to measure than when grown horizontally on quartz sand. When growing the test species on filter paper, some paper stuck to the root making the root DM measurement inaccurate. A disadvantage of the present bioassays is that they need a rather large amount of substrate/liquid. We used 45 ml in Method 1 and 30 ml in Method 2. This is much more than can be applied when using bioassay as a part of a separation with HPLC, where 1–2 ml is an appropriate amount. On the other hand, relatively large volumes of solutions/extracts can be important in developing a sensitive bioassay. Weidenhamer et al. (1987) reported that the number of seeds relative to the solution volume used in a seed germination bioassay influenced results. They found that the amount of ferulic acid available to each seed influenced germination, rather than the concentration of the chemical in the solution. The present bioassay method on filter paper also gave low standard deviation of the mean, indicated by a statistically significant difference of one to two millimeters.

Many investigators (e.g., Shilling et al., 1985; Kato-Noguchi et al., 1994) used evaporation in vacuo for increasing concentration of the extracts without testing the effect of the evaporator treatment. We did not find any effects of such a treatment on the activity of the extracts in the present investigation. The most important question when using an evaporator is probably whether the boiling points of the active substances are lower or higher than that of the extraction liquid, in our case water. If the boiling points of the active substances are lower than that of the extraction liquid, there is a possibility of losing the active substances in the concentration process. Additionally evaporator treatments may cause changes in chemical structures and phytotoxicity of allelochemicals.

It is important to confirm the results from the laboratory bioassays in field studies as proposed by Fuerst and Putnam (1983). It is in this context an advantage if the bioassay reflects the field conditions as well as possible. Krogmeier and Bremner (1989) tested the effects of phenolic acids on germination and seedling growth on germination paper and in sand. Although the phenolic acids affected germination and growth on germination paper, no such effects were detected in soil. On the other hand, indications of delayed germination was found by Moore et al. (1994) in a study of cover crop mulches. Under certain conditions the number of emerged *Amaranthus retroflexus* L. seedlings was reduced compared with bare soil in early summer, while no differences in total number of seedlings over the entire season were observed. The observed delay in germination under field conditions could additionally be a response of changed physical conditions, e.g., lower soil temperature, as well as a response of allelopathic compounds. Obviously, there are needs for developing field techniques for separating the effects of allelopathy from other factors. Rape and rye were not included in the present investigations to compare phytotoxicity of the species. Although bioassays using water extracts from plants are useful to prove the existence of allelopathic substances in them, these effects often disappear under field conditions (Etherington, 1975) due to adsorption to soil particles, decomposition and leaching (Almeida, 1985). Additionally varying phytotoxicity between species is dependent on environmental factors (Mason-Sedun and Jessop, 1989) and age (Wardle et al., 1993) and these important factors were not included in the present investigations.

In conclusion, bioassay methods, test species, light conditions, and osmotic potential and interactions between these factors clearly influence the sensitivity and the results of the bioassays. Confounding the effects on germination and growth may lead to misinterpretation of the results. It is important to realize that the bioassay performance and its relationship to field conditions are vital for confirming the results of bioassays by field test. Therefore, it may be necessary to optimize the conditions in the bioassay for each case of study.

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GREEN LEAF VOLATILES AS ANTIAGGREGANTS FOR THE MOUNTAIN PINE BEETLE, *Dendroctonus ponderosae* HOPKINS (COLEOPTERA: SCOLYTIDAE)

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Abstract—We tested the hypothesis that green leaf volatiles act as antiaggregants for the mountain pine beetle (MPB), *Dendroctonus ponderosae* Hopkins. In coupled gas chromatographic-electroantennographic detection (GC-EAD) analysis MPB antennae responded to 30 ng doses of all six-carbon green leaf alcohols tested [1-hexanol, (*E*)-2-hexen-1-ol, (*Z*)-2-hexen-1-ol, (*E*)-3-hexen-1-ol, and (*Z*)-3-hexen-1-ol], but not to the aldehydes, hexanal or (*E*)-2-hexenal, or to alcohol or aldehyde homologues with more or fewer than six carbon atoms. In field trapping experiments a blend of green leaf alcohols [1-hexanol, (*Z*)-2-hexen-1-ol, (*E*)-3-hexen-1-ol and (*Z*)-3-hexen-1-ol] effectively disrupted the response to attractive semiochemicals; a blend of the aldehydes hexanal and (*E*)-2-hexenal was inactive. The two best disruptants, (*E*)-2-hexen-1-ol and (*Z*)-3-hexen-1-ol, reduced catches of both sexes to levels not significantly different from catches in unbaited control traps. They also reduced the attack on trees baited with attractive MBP pheromones to a level not significantly different from that on unbaited control trees. Neither of the clerid predators captured, *Enoclerus sphegeus* (F.) nor *Thanasimus undatulus* (Say), was repelled by green leaf volatiles. Our results suggest that green leaf alcohols are promising disruptants which may be used to supplement the antiaggregation pheromone, verbenone, in protecting single high-value trees as well as carefully selected stands with low-level populations of MPBs.

Key Words—Mountain pine beetle, *Dendroctonus ponderosae*, Coleoptera, Scolytidae, antiaggregant, verbenone, green leaf volatiles, 1-hexanol, (*E*)-2-hexen-1-ol, (*E*)-3-hexen-1-ol, (*Z*)-2-hexen-1-ol, (*Z*)-3-hexen-1-ol.

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INTRODUCTION

The mountain pine beetle (MPB), *Dendroctonus ponderosae* Hopkins, is the most destructive insect pest of pines in western North America (Furniss and Carolin, 1977). A number of strategies and tactics have been developed and implemented for integrated MPB management in British Columbia (Borden and Lindgren, 1988; Maclauchlan and Brooks, 1994). However, there is a need for environmentally acceptable tactics, such as the use of antiaggregants, to protect high value commercial timber stands, as well as stands in such areas as campgrounds, parks, and riparian zones (Amman et al., 1989, 1991; Lindgren et al., 1989; Gibson et al., 1991; Shea et al., 1992; Amman, 1994).

Most research with antiaggregants for the MPB has focused on the antiaggregation pheromone verbenone (Lindgren and Borden, 1989, 1993; Amman, 1994; Borden, 1996). Attempts to develop verbenone as a reliable tool for controlling MPBs have utilized either aerially dispersed verbenone-impregnated pellets (Shea et al., 1992), or bubble-cap release devices affixed to trees at 10 m centers throughout a stand (Amman et al., 1989; Lindgren et al., 1989). While both methods are sometimes effective (Amman et al., 1989, 1991; Lindgren et al., 1989; Shea et al., 1992), results have been inconsistent from year to year in lodgepole pine forests (Gibson et al., 1991; Shea et al., 1992), and none of the trials in ponderosa pine forests has yielded significant results (Bentz et al., 1989; Lister et al., 1990; Gibson et al., 1991). Verbenone does appear to have the ability to protect single, high-value trees from attack (Borden and Lindgren, 1988; Shore et al., 1992).

Recent research has supported the hypothesis that combinations of semiochemicals can be much more effective than a single disruptant (Borden, 1996). Although research on combinations of disruptants has focused on semiochemicals produced by con- or heterospecific bark beetles, it may be possible to integrate "resistant" and nonhost kairomones into an antiaggregant blend.

Changes in host suitability could be detected through kairomones such as 4-allylanisole (also known as methyl chavicol or estragole) (Hobson 1995). Stark et al. (1968) reported a 70% drop in 4-allylanisole content in ozone-damaged ponderosa pines which were preferentially attacked by western pine beetles, *Dendroctonus brevicomis* LeConte. 4-Allylanisole has since been found to disrupt the response to attractant-baited traps by six *Dendroctonus* and one *Ips* species (Hayes et al. 1994; Hayes and Strom, 1994; Werner 1995; Hobson 1995).

One particularly ubiquitous group of nonhost chemicals, the green leaf volatiles (GLVs), are also promising candidates for improved disruptant blends. GLVs are six carbon alcohols, aldehydes, and derivative esters commonly found in green plants (Visser et al., 1979; Visser, 1986; Whitman and Eller, 1990). Formed through oxidative degradation of leaf lipids, GLVs are released contin-

ually by leaves (Visser and Avé, 1978). They can function as attractive kairomones (Visser et al., 1979; Guerin et al., 1983; Kamm and Buttery, 1983; Katsoyannos and Guerin, 1984), synergistically enhance attraction to sex pheromones (Dickens et al., 1990), or act as synomones in tritrophic interactions in which natural enemies of herbivores are attracted to GLVs released by herbivore-damaged plants (Turlings et al., 1990, 1993; Whitman and Eller, 1990; Ramachandran and Norris, 1991).

GLVs have also shown disruptive activity. Although a blend of GLVs attracts the Colorado potato beetle, *Leptinotarsa decemlineata* Say, to host plants, none is attractive alone, and even a slight change in the ratio of GLVs can decrease attraction (Visser and Avé, 1978). Two compounds, not in the attractive blend, were also active in electrophysiological tests and disrupted positive anemotaxis to potato odor; (*Z*)-2-hexen-1-ol, present in potato foliage, was a weak disruptant, whereas a compound absent from potato foliage, (*Z*)-3-hexen-1-ol (Visser et al., 1979), was a strong disruptant (Visser and Avé, 1978). Similarly, hexanal and 1-hexanol disrupted the response of three species of bark beetles, the southern pine beetle, *Dendroctonus frontalis* Zimmerman, the eastern fivespined ips, *Ips gradicollis* (Eichhoff), and the small southern pine engraver, *Ips avulsus* (Eichhoff), to traps baited with attractant semiochemicals. (Dickens et al. 1991, 1992, 1993, 1995).

Our objective was to test GLVs as disruptants that could potentially enhance the disruptant activity of verbenone as an operational tool against the MPB.

METHODS AND MATERIALS

Electrophysiological Studies. Eighteen synthetic chemicals, GLVs or their homologues containing more or fewer carbon atoms (Table 1), were analyzed (30 ng each in 1 μl of hexane) by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn et al., 1975). MPB-infested lodgepole pine bolts were collected near Princeton, B.C., and placed in cages in the laboratory. Emerged beetles were removed from cages daily and used immediately in GC-EAD analysis. The apparatus employed a Varian 3400 gas chromatograph equipped with a DB-5-coated, fused silica column (30 m $\times$ 0.32 mm I.D.) (J & W Scientific, Folsom CA). The recording electrode was placed into the antennal club of an intact immobilized beetle with a micromanipulator (Type M, Leitz Canada, Willowdale, Ontario), and the indifferent electrode was inserted into the head. Both *exo*- and *endo*-brevicomin (10 ng each in 1 μl of hexane) were used as standard stimuli in all tests. Beetles that did not respond to brevicomin were discarded.

Field Trapping Experiments. Four randomized, complete block trapping experiments were conducted in 1993 and 1994 in stands of lodgepole pine,

TABLE 1. CHEMICALS TESTED AT 30 ng EACH BY GC-EAD ANALYSIS WITH THE MOUNTAIN PINE BEETLE

Chemical	Source[a]	Purity (%)[b]	Antennal response (+)
1-propanol	Aldrich	99	
1-butanol	Anachemia	99	
1-pentanol	Sigma	99	
1-hexanol	Sigma	98	+
(*Z*)-2-hexen-1-ol	Aldrich	96	+
(*E*)-2-hexen-1-ol	Aldrich	95	+
(*Z*)-3-hexen-ol	Aldrich	98	+
(*E*)-3-hexen-1-ol	Aldrich	98	+
1-heptanol	Aldrich	99	
1-octanol	Aldrich	99	
1-nonanol	Sigma	98	
1-decanol	Sigma	98	
hexanal	Aldrich	98	
(*E*)-2-hexenal	Aldrich	99	
heptanal	Aldrich	95	
octanal	Aldrich	99	
nonanal	Aldrich	95	
decanal	Sigma	99	

[a]Chemical sources: Aldrich Chemical Co., Milwaukee, WI; Anachemia Chemical Co., Vancouver, BC; Sigma Chemical Co., St. Louis, MO.
[b]Purity as listed by manufacturer.

Pinus contorta var. *latifolia* Engelmann, near Princeton, British Columbia. Locations, dates, and numbers of replicates are given in Figures 1–4. All experiments utilized 12-unit multiple funnel traps (Lindgren, 1983) hung from ropes or poles. Traps were deployed in lines with 25 m between traps and lines. All chemicals, sources, release devices, and release rates are listed in Table 2. The GLVs tested were chosen because they were often referred to in the literature, and were cheap and easy to obtain. Release rates of verbenone were lower than optimal to allow the effects of GLVs to be detected.

Except where otherwise stated, three control treatments were used to assess the effectiveness of candidate disruptants: (1) attractive control, consisting of a three-component MPB lure (Borden et al., 1987a); (2) disruptant control, consisting of a MPB lure plus verbenone; and (3) unbaited trap.

Experiment 1 compared the disruptive effect of hexanal (Dickens et al., 1992) vs. hexanal plus a blend of four other GLVs (released separately) (Table 2). The aim of Experiment 2 and 3 was to discern the most active components of the GLV blend. Experiment 2 compared the disruptive effect of green leaf aldehydes, alcohols, or both, added to MPB lures. Because the green leaf

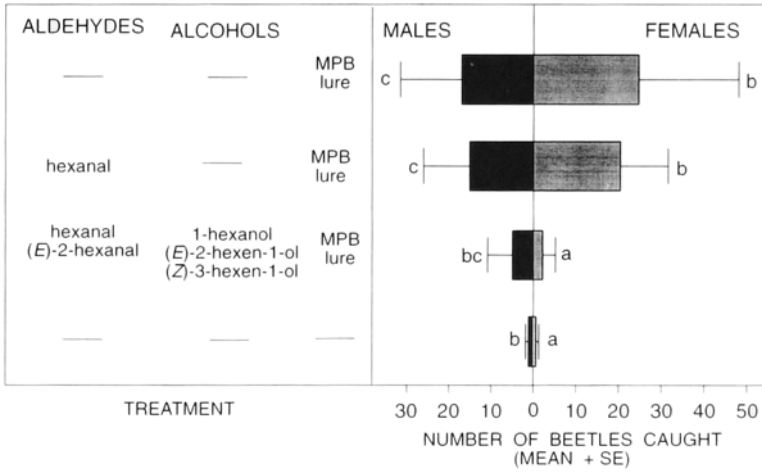

FIG. 1. Response of *Dendroctonus ponderosae* in Experiment 1 to multiple funnel traps baited with MPB lures alone, and in combination with hexanal or hexanal plus four other green leaf volatiles released from separate devices (Table 2). McNulty Ck., near Princeton, B.C., Aug. 22–31, 1993, N = 10. For each sex, bars associated with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsch Multiple Q-test, $P < 0.05$.

alcohols were the most repellent GLVs in Experiment 2, they were tested individually in Experiment 3; there was no MPB lure control in this experiment. In Experiment 4, the two best individual GLVs from Experiment 3 were tested with or without verbenone, to determine if they could enhance the repellency of verbenone.

Captured MPBs were collected within a week and stored in a freezer until counted and sexed. The numbers of two species of clerid beetles captured were also recorded.

Tree-Baiting Experiment. The two most potent GLVs, (*E*)-2-hexen-1-ol and (*Z*)-3-hexen-1-ol, were tested in Experiment 5 for their ability to deter attack on trees baited with MPB tree-baits (Phero Tech Inc., Delta, B.C.) releasing the attractive pheromones *trans*-verbenol and *exo*-brevicomin. The experiment was set up on July 19, 1995 with randomized complete blocks in Cutting Permit No. 18 (Weyerhaeuser Canada Ltd., Princeton, B.C.) in a mature stand of predominately lodgepole pine attacked at a moderate level by the MPB. Experimental trees were 25 m apart, and included those left as unbaited controls (11 trees), baited with MPB tree-baits (nine trees), or baited with MPB tree baits plus the two GLVs (ten trees) (Table 2). All release devices were stapled ≥ 2 m high on the north face of the tree. Mean diameters ($\pm$SE) at 1.3 m high

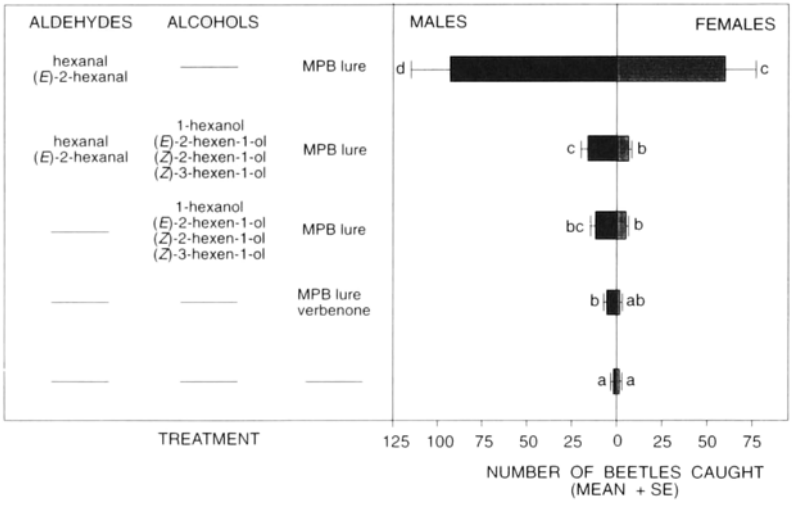

FIG. 2. Response of *Dendroctonus ponderosae* in Experiment 2 to multiple funnel traps baited with MPB lures in combination with green leaf alcohols, aldehydes, or both (Table 2). Willis Ck., near Princeton, B.C., July 11–23, 1994, N = 20. For each sex, bars associated with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsch Multiple Q-test, $P < 0.05$.

for the trees in the three respective treatments were 25.4 ± 0.8, 25.5 ± 2.1, and 24.9 ± 1.3 cm.

The experiment was assessed on July 25, and August 10, 1995. On both dates the MPB attack density was counted in two 20 × 40 cm panels at eye level on the east and west faces of each experimental tree. At the second assessment all trees ≥ 15 cm in diameter within 5 m of each experimental tree were classed as not attacked, attacked with 1–4 attacks in the two 20 × 40 cm sample areas (< 31.25 attacks per m^2), or mass-attacked with ≥ 5 attacks in the two sample areas (≥ 31.25 attacks per m^2).

Statistical Analysis. In all cases $\alpha = 0.05$. To satisfy criteria for normality and homoscedasticity, trap catch and attack density data were transformed by $\log_{10}(x + 1)$ (Zar, 1984). ANOVA (GLM procedure, SAS Institute Inc., 1985), and the Ryan-Einot-Gabriel-Welsch Multiple Q-test (REGWQ procedure, SAS Institute Inc. 1985; Day and Quinn 1989) were used to determine differences between means in all experiments. Orthogonal contrasts (SAS Institute Inc., 1985) were also used to compare treatment means to the blank trap for low catches of clerid predators in Experiment 2. In Experiment 3, nonparametric Mann-Whitney-Wilcoxon tests (Zar, 1984) were used to test the hypothesis that trap catches to two semiochemical treatments containing green leaf alcohols

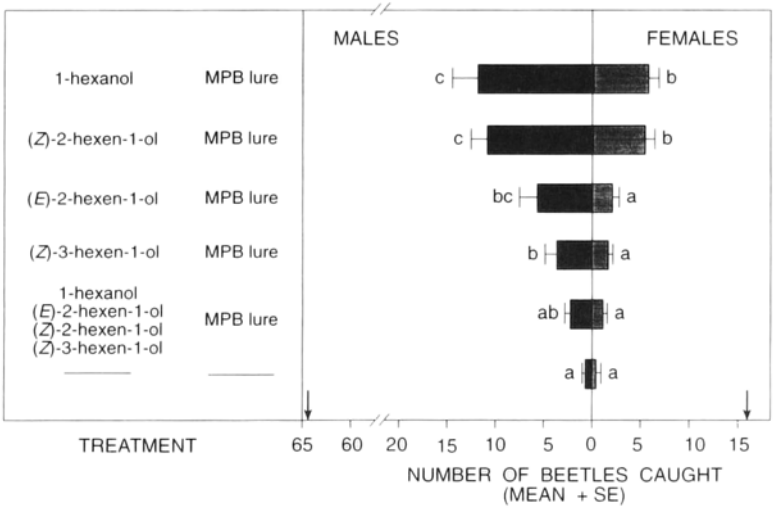

FIG. 3. Response of *Dendroctonus ponderosae* in Experiment 3 to multiple funnel traps baited with MPB lures in various combinations with green leaf alcohols. Willis Ck., near Princeton, B.C., July 23–Aug. 1, 1994, N = 16. Arrows on x-axis indicate mean catches (N = 2) in monitoring traps adjacent to the experiment, serving as a partial attractive control. For each sex, bars associated with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsch Multiple Q-test, $P < 0.05$.

were significantly lower than catches in two nearby MPB monitoring traps containing attractive lures. The proportions of trees within 5 m of treatment trees in Experiment 5 that were attacked at any density or were mass-attacked were compared by chi-square tests for multiple proportions (Zar, 1984).

RESULTS AND DISCUSSION

Electrophysiological Studies. In GC-EAD analyses MPB antennae responded to all six-carbon alcohols tested, including 1-hexanol, (*E*)-2-hexen-1-ol, (*Z*)-2-hexen-1-ol, (*E*)-3-hexen-1-ol, and (*Z*)-3-hexen-1-ol (Table 1). Responses (0.1–0.3 mV) were reproduced in at least two tests. These six-carbon alcohols were tested in field experiments, except for (*E*)-3-hexen-1-ol which is too expensive to be used operationally. At the 30 ng doses tested, MPB antennae did not respond to any of the six-carbon aldehydes or to homologous alcohols or aldehydes containing more or fewer carbon atoms.

Field Trapping Experiments. Hexanal was an ineffective disruptant when released at 1.2 mg per 24 hr in Experiment 1 (Figure 1). This result differs from

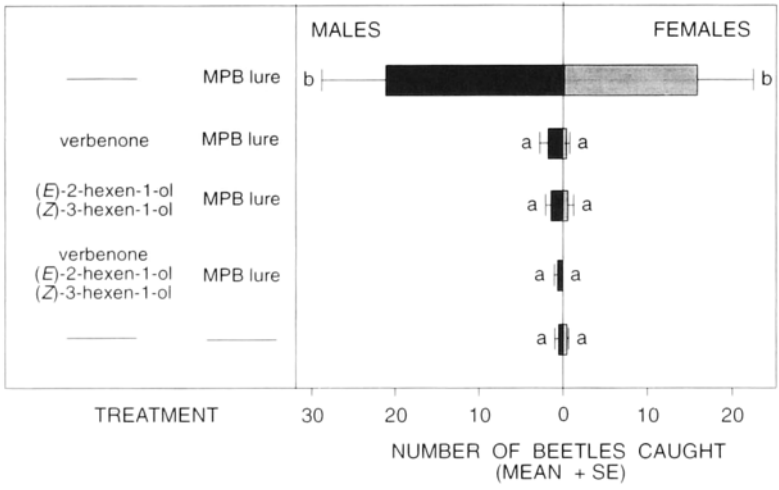

FIG. 4. Response of *Dendroctonus ponderosae* in Experiment 4 to multiple funnel traps baited with MPB lures alone or in combination with two green leaf alcohols, verbenone, or both. Willis Ck., near Princeton, B.C., Aug. 12–16, 1994, N = 10. For each sex, bars associated with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsch Multiple Q-test, $P < 0.05$.

the finding of Dickens et al. (1992) that hexanal was a more effective disruptant than 1-hexanol for three other species of bark beetles. In other experiments (results not shown) hexanal released at rates up to 7.0 mg per 24 hr also had no significant disruptive effect. The lack of an antennal response to hexanal is in agreement with its behavioral inactivity. The blend of GLVs in Experiment 1, however, strongly reduced catches of both sexes, with catches of females being not significantly different from those in unbaited traps. Experiment 2 demonstrated that a green leaf alcohol blend comprising 1-hexanol, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, and (*Z*)-2-hexen-1-ol (the latter not tested in Experiment 1), was competitive with verbenone as an antiaggregant, whereas the aldehydes, hexanal and (*E*)-2-hexenal were poor disruptants and did not enhance the effect of the alcohols (Figure 2).

(*Z*)-3-hexen-1-ol and (*E*)-2-hexen-1-ol in Experiment 3 were the most potent green leaf alcohol antiaggregant components of the mixture from Experiment 2 (Figure 3). Each of these alcohols alone disrupted the response of both sexes of MPB to the same level as a blend of all four alcohols, and reduced catches of females to levels not significantly different from those in unbaited traps. Although MPB lure controls were not included in Experiment 3, mean catches ($\pm$SE) (N = 2) in nearby monitoring traps baited with MPB lures were 64.0 $\pm$ 13.0 males and 16.5 $\pm$ 2.5 females, respectively (Figure 3, arrows). These

TABLE 2. DESCRIPTION OF CHEMICALS EMPLOYED IN FOUR RANDOMIZED-BLOCK TRAPPING EXPERIMENTS (EXPERIMENTS 1–4) AND ONE TREE-BAITING EXPERIMENT (EXPERIMENT 5) FOR THE MOUNTAIN PINE BEETLE (MPB)

Semichemical[a]	Source[b]	Experiment	Release device	Release rate (mg per 24 hr)
MPB lure				
myrcene	P	1–4	1 closed polyethylene bottle (15 ml)	18.0
trans-verbenol	P	1–5	1 bubble cap	1.0
exo-brevicomin	P	1–4	1 flex lure	0.05
		5	1 flex lure	0.3
verbenone	P	2	2 bubble caps	1.2
		4	1 bubble cap	0.6
hexanal	A	1	1 capillary tube (1 mm I.D.)	1.2[c]
			1 bubble cap	13.0
(*E*)-2-hexenal	A	1	1 capillary tube (1 mm I.D.)	1.3[c]
	B	2	1 bubble cap	13.0
1 hexanol	A	1,2	1 open polyethylene microcentrifuge tube (400 μl)	2.1[c]
	S	3	1 bubble cap	3.8
(*E*)-2-hexen-1-ol	A	1,2	1 open polyethylene microcentrifuge tube (400 μl)	1.8[c]
	B	3–5	1 bubble cap	3.8
(*Z*)-2-hexen-1-ol	B	2,3	1 bubble cap	3.8
(*Z*)-3-hexen-1-ol	A	1,2	1 open polyethylene microcentrifuge tube (400 μl)	1.3[c]
	B	3–5	1 bubble cap	3.8

[a] All green leaf volatiles stabilized with 1.2% (wet weight) Ethanox® 330 antioxidant, Ethyl Chemicals Group, Baton Rouge, LA.

[b] Symbols for sources as follows: P = Phero Tech Inc., Delta, BC; A = Aldrich Chemical Company, Milwaukee, WI; S = Sigma Chemical Company, St. Louis, MO; B = Bedoukian Research Inc., Danbury, CT; Purities as in Table 1, except for, (*E*)-2-hexen-1-ol (95%), (*Z*)-2-hexen-1-ol (92%), (*Z*)-3-hexen-1-ol (97%), and (*E*)-2-hexenal (98%).

[c] Determined in laboratory at 24°C. All other release rates determined by Phero Tech, Inc. at 22°C.

catches were significantly higher (one-tailed Mann-Whitney-Wilcoxon tests, $P < 0.05$) than those to traps baited with MPB lures plus either 1-hexanol or (*Z*)-2-hexen-1-ol, indicating that these two alcohols also have disruptive properties. A combination of (*Z*)-3-hexen-1-ol and (*E*)-2-hexen-1-ol in Experiment 4 (Figure 4) had the same disruptive power as verbenone, reducing catches of male and female MPBs by 94% and 96%, respectively, compared to the attractive MPB lure control.

The reduction in trap catches caused by GLVs, ranging from 71% in Experiment 1 to 96% in Experiment 4, compares favorably with results of other

investigators, particularly when release rates (Table 2) are considered. When hexanal was released at 0.3 ml per 24 hr from traps also containing attractants, trap catches of *D. frontalis*, *I. grandicollis*, and *I. avulsus* were reduced by 46, 54, and 69%, respectively (calculated from tabular data in Dickens et al. 1992). 4-Allylanisole also caused significant reductions in response by *D. frontalis* (about 25–38%, calculated from Figures 1 & 2 in Hayes et al., 1994), and a 77% reduction in response by *D. ponderosae* (Hayes and Strom, 1994), but the release rates were very high, 160–1237 and 160 mg per 24 hr, respectively. At a similarly high release rate of 0.5 ml per 24 hr, 4-allylanisole caused reductions in response to attractant-baited traps by *D. brevicomis*, *D. ponderosae*, and *Ips pini* (Say) of 60, 71, and 29%, respectively (Hobson, 1995).

Tree-Baiting Experiments. At both assessment dates, the mean attack density by MPBs in trees baited with tree-baits plus GLVs was significantly lower than that on trees baited with MPB baits alone, and no different from that on unbaited control trees (Table 3). By the second assessment, nine of ten trees baited with MPB baits plus GLVs, and seven of 11 unbaited trees were attacked, but only three trees in each category were mass-attacked. In contrast, eight of

TABLE 3. EFFECT ON ATTACK BY *Dendroctonus ponderosae* IN EXPERIMENT 5 CAUSED BY AFFIXING DEVICES RELEASING TWO GLVS, (*e*)-2-HEXEN-1-OL AND (*Z*)-3-HEXEN-1-OL, ON TREES ALSO BAITED WITH MPB TREE-BAITS. CUTTING PERMIT NO. 18, APPROXIMATELY 20 KM SOUTHWEST OF PRINCETON, B.C., SET UP JULY 19, 1995

		Attack on treatment trees[b]				Attack on trees ≥ 15.0 cm diam. within 5 m of experimental trees[b]		
Assessment date	Treatment[a]	N	Number attacked	Number mass attacked	Attack density per m^2 ($\bar{x} \pm$ SE)	N	Percent attacked	Percent mass attacked
July 25	MPB	9	7	7	68.1 ± 14.9a	—	—	—
	MPB+GLVs	10	4	2	13.1 ± 7.9b	—	—	—
	Unbaited	11	2	1	6.3 ± 5.7b	—	—	—
August 10	MPB	9	9	8	84.7 ± 13.6a	33	51.5a	27.3a
	MPB+GLVs	10	6	3	22.5 ± 8.9b	46	30.4ab	19.6a
	Unbaited	11	4	3	24.4 ± 12.5b	55	16.4 b	10.9a

[a] MPB tree-baits (Phero Tech) contain *trans*-verbenol and *exo*-brevicomin. Release rates of these pheromones and the GLVs are as given in Table 2.

[b] Attack densities for each assessment date followed by the same letter are not significantly different, Ryan-Einot-Gabriel-Welsch Multiple Q-test, $P < 0.05$. Percents within a column followed by the same letter are not significantly different, chi-square test for multiple proportions, $P < 0.05$.

nine trees treated with MPB baits alone were mass-attacked. The GLV treatment reduced the total proportion of "spillover" attacks on trees surrounding experimental trees to a level intermediate between that for trees baited with MPB baits alone and that for unbaited control trees. It had no effect on the proportions of mass-attacked trees (Table 3). The GLVs were as effective as the antiaggregation pheromone, verbenone, in reducing attack density on trees baited with MPB pheromones, but not as effective in reducing spillover attacks (Borden and Lindgren, 1988).

The antennal responses (Table 1) and behavioral activity induced by green leaf alcohols in field trapping and baited tree experiments (Figures 1–4, Table 3) supports the hypothesis of Dickens et al. (1991, 1992) that GLVs are used by scolytids in host selection. GLVs may direct MPBs away from nonhost trees and may also help them to detect and fly above a deciduous understorey (Safranyik et al., 1992), possibly explaining why MPB tree baits placed on lodgepole pines within a dense understorey of Sitka alder, *Alnus sinuata* (Regel) Rydb., were ineffective at inducing mass-attack (Borden et al., 1986).

Associated Insects. Both *Enoclerus sphegeus* (F.) and *Thanasimus undatulus* (Say), clerid predators of the MPB (Furniss and Carolin, 1977), were captured in large numbers in Experiment 2 (Figure 5). *E. sphegeus* responded

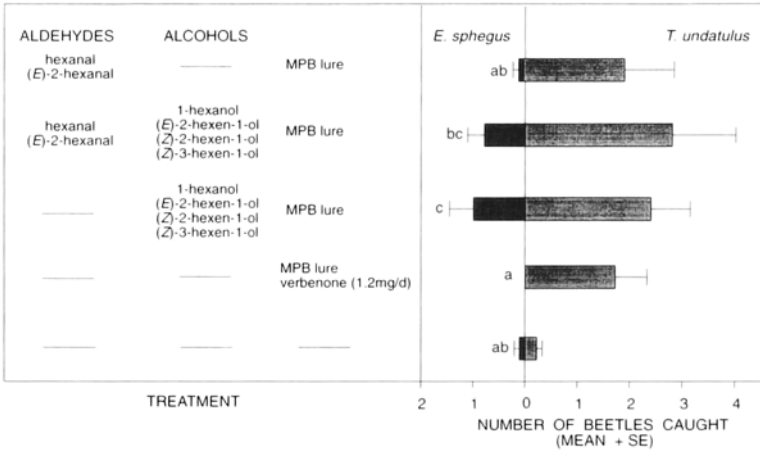

FIG. 5. Response of 20 *Enoclerus sphegeus* and 90 *Thanasimus undatulus* in Experiment 2 (Fig. 2) to multiple funnel traps baited with MPB lures in combination with verbenone, or green leaf alcohols, aldehydes, or both. Bars for *E. sphegeus* associated with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsch Multiple Q-test, $P < 0.05$, $N = 10$. Orthogonal contrasts on *T. undatulus* catches indicated that all semiochemical-baited traps caught significantly more beetles than the unbaited traps ($P = 0.004$).

significantly only to traps baited with MPB lures plus GLV alcohols, whereas all semiochemical-baited traps caught significantly more *T. undatulus* than unbaited traps. Neither *E. sphegeus* nor *T. undatulus* were repelled by GLVs, and *E. sphegeus* may even have been slightly attracted by six-carbon alcohols. Similarly, 4-allylanisole did not reduce the response of *Thanasimus dubius* (F.), or *Temnochila chlorodia* (Mannerhein) to the attractant pheromones of their prey (Hayes et al., 1994 Hayes and Strom, 1994). In contrast, the response by predatory beetles, *Rhizophagus depressus* (F.), to ethanol was disrupted by nonhost volatiles (Schroeder, 1992).

The responses of clerid predators (Figure 5) are consistent with the hypothesis that semiochemicals associated with prey are used as host-finding kairomones by entomophagous insects (Borden 1982). *E. sphegeus* was previously shown to respond to *exo*-brevicomin (Borden et al. 1987b), a pheromone found in numerous species of scolytids. *T. undatulus* responds strongly to frontalin, another common aggregation pheromone in scolytids (Borden 1982).

Practical Implications. Of the candidate antiaggregants tested for the MPB, verbenone and the green leaf alcohols appear to be the most promising. Ipsdienol, the principal aggregation pheromone of the heterospecific competitor, *I. pini*, significantly disrupts response of MPBs to attractive baits (Hunt and Borden 1988; Wilson 1995), but it is expensive and may not be viable operationally except for protection of single, high-value trees. Green leaf alcohols have the advantage of being cheap and easy to obtain, but more work is needed to bring them to an operational level for use against the MPB. A disruptant blend of verbenone with GLVs (Dickens et al. 1993, 1995), and possibly with interspecific synomones, and resistant host or nonhost kairomones, may prove to be a reliable tool for operational protection of trees and stands. Because 4-allylanisole can protect trees from attack by the southern pine beetle (Hayes and Strom, 1995), it could be a component of an operational blend. A disruptant blend could be an even more powerful tool if used with attractants in a "push-pull" tactic (Lindgren and Borden 1993; Borden 1995).

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PHLOROTANNIN-PROTEIN INTERACTIONS

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Abstract—Tannins are one of the most broadly distributed types of plant secondary compounds, and have been the focal point for many studies of plant/herbivore interactions. Tannins interact strongly with proteins, so that the fate and effects of ingested tannins are in part dependent on the mode of interaction of the tannin with dietary and endogenous proteins in an herbivore's gut. We investigated the factors affecting the precipitation of proteins by phlorotannins from three species of marine brown algae: *Carpophyllum maschalocarpum*, *Ecklonia radiata*, and *Lobophora variegata*. Phlorotannins were precipitated by proteins in a pH-dependent and concentration-dependent fashion. Precipitation also varied as a function of the presence of reducing agent, the type of phlorotannin or protein used, and the presence of organic solvents such as hydrogen bond inhibitors. Of particular significance was the ability of some phlorotannins to oxidize and form covalent bonds with some proteins. In contrast, under similar experimental conditions three types of terrestrial tannins (procyanidins, profisetinidins, and gallotannins) apparently did not form covalent complexes with proteins. Our results suggest several ways in which the biological activity of phlorotannins may vary as a function of the properties of the gut environment of marine herbivores. Moreover, we identify specific structural characteristics of phlorotannins which affect their tendency to oxidize, and thus, their potential effects on marine herbivores.

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Key Words—Tannin-protein interaction, tannins, hydrogen bonding, protein precipitation, marine brown algae, phlorotannins, *Carpophyllum maschalocarpum*, *Ecklonia radiata*, *Lobophora variegata*.

INTRODUCTION

Tannins, or polymeric phenolics, are one of the most broadly distributed types of plant secondary metabolites in terrestrial (Harborne, 1991) and marine (Ragan and Glombitza, 1986) plants. Tannins have important effects on both marine and terrestrial herbivores, but there is substantial variation in the biological activities of tannins against different herbivores (Bernays et al., 1989; Steinberg, 1993; Appel, 1993; Boettcher and Targett, 1993). In part this variation is because tannins do not have a single biological activity, but may act as feeding deterrents (Steinberg, 1988; Clausen et al., 1990); digestibility reducing agents (Robbins et al., 1987; Boettcher and Targett, 1993); or toxins (Bernays et al., 1980).

The variation in biological activity of tannins is due in part to variation in tannin structure (Zucker, 1983). For example, terrestrial tannins bind proteins selectively (Hagerman and Butler, 1981), and some mammals utilize that selectivity by secreting salivary tannin-binding proteins which neutralize specific tannins (Hagerman and Robbins, 1993). Even minor structural variation, such as stereoisomerism, affects the activity of some terrestrial proanthocyanidins as feeding deterrents (Clausen et al., 1990). The ability of phlorotannins to inhibit protein assimilation by an herbivorous fish varied with the molecular weight of the tannin (Boettcher and Targett, 1993). Phlorotannins from different species of brown algae also varied in their activity as feeding deterrents for marine herbivores (Steinberg and van Altena, 1992; van Altena and Steinberg, 1992) although the structural elements leading to the variation in activity were not identified.

The variation in the biological activity of tannins can also be attributed to variations in the gut physiology and biochemistry of various herbivores. For example, the ability of tannins to precipitate proteins is dependent on pH, the presence of surfactants, and the concentration of metal ions (Hagerman and Butler, 1978; Martin and Martin, 1984; Tugwell and Branch, 1992). All of these factors affect how tannins affect herbivores, and how herbivores can minimize the detrimental effects of tannins on ecological or evolutionary time scales (Bernays et al., 1989; McArthur et al., 1991).

The ability of tannins to precipitate protein has been examined in many studies. However, with few exceptions (Tugwell and Branch, 1992; Boettcher and Targett, 1993), most of the work on interactions between proteins and tannins has been done using terrestrial tannins (Hagerman, 1992). It may be inappropriate to draw conclusions about marine herbivore/phlorotannin interactions based on knowledge about terrestrial tannins. Phlorotannins (1–4, Figure

1), which are known only from the Phaeophyta (brown algae) (Ragan and Glombitza, 1986), are structurally quite unlike terrestrial tannins (5, 6, Figure 1). Furthermore, the gut morphology, physiology, and biochemistry of marine herbivores are quite unlike those of terrestrial herbivores (Horn, 1989). We have therefore conducted experiments designed to define the parameters that control

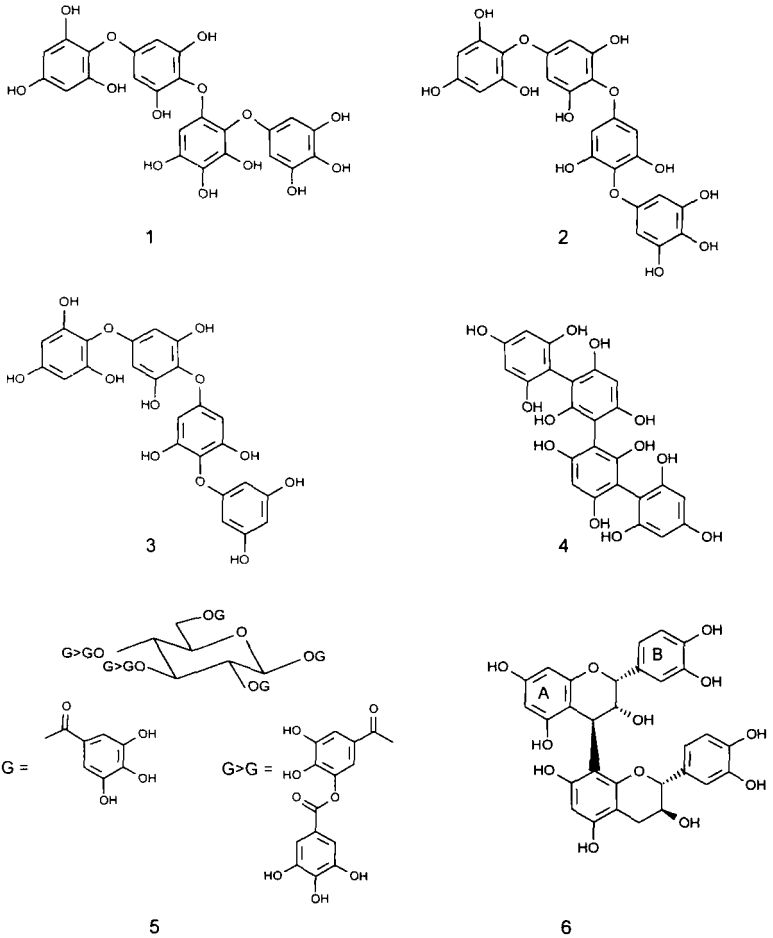


FIG. 1. Structural formulae of various tannins. 1, tetrafuhalol A, is a fuhalol-type phlorotannin; 2, hydroxytetraphlorethol, is a hydroxyphlorethol-type phlorotannin; 3, tetraphlorethol C, is a phlorethol-type phlorotannin; 4, tetrafucol A, is a fucol-type phlorotannin; 5, heptagalloyl glucose, is a gallotannin; 6, epicatechin-4β → 8-catechin (procyanidin B-1), is a procyanidin-type condensed tannin.

precipitation of phlorotannins by proteins. Phlorotannins from three species of brown algae and several proteins were used to evaluate structure-activity relationships. We examined the effects of pH, of hydrogen bonding solvents, and of detergents and hydrophobic solvents on the interaction between phlorotannins and proteins.

In the course of our study, it became clear that oxidation reactions were an important feature of phlorotannin chemistry. It has been postulated that oxidation of tannins may be crucial to their biological activity (Appel, 1993), and it has been demonstrated that tannic acid precipitates protein more effectively in the presence of oxidants (Leatham et al., 1980). However, we believe that our work with phlorotannins is the first clear demonstration of a role for spontaneous oxidation in the formation of tannin-protein complexes. We were unsuccessful in our attempt to demonstrate similar reactions for several representative terrestrial tannins.

METHODS AND MATERIALS

Reagents

Phlorotannins were purified from *Ecklonia radiata* (Order Laminariales) and *Carpophyllum maschalocarpum* (Order Fucales) using methods developed by Ragan and Glombitza (1986) as modified by Steinberg and van Altena (1992). High (>100,000 amu) and moderate (5,000–100,000 amu) molecular weight phlorotannins from *Lobophora variegata* (Order Dictyotales) (Boettcher and Targett, 1993) were provided by Nancy M. Targett (University of Delaware, Lewes, Delaware, 1995). Terrestrial tannins were purified from *Sorghum* grain, from commercial tannic acid or from crude commercial quebracho tannin (Hagerman and Butler, 1980b; Asquith and Butler, 1985; Hagerman and Klucher, 1986). Phlorotannin preparations were comprised of polydisperse tannins with molecular weights ranging from 6,000–100,000 (Van Altena and Steinberg, 1992; Boettcher and Targett, 1993). Condensed tannins from *Sorghum* grain or quebracho did not contain any low molecular weight phenolics (Hagerman and Butler, 1980b). Purified tannic acid is a mixture of polygalloyl esters ranging from pentagalloyl glucose to decagalloyl glucose (Hagerman and Klucher, 1986).

Proteins and 2,4-dimethoxybenzaldehyde (DMBA) were obtained from Sigma Chemical Co., St. Louis, MO. Radiolabeled bovine serum albumin was prepared by reacting $Na^{125}I$ with bovine serum albumin in the presence of Chloramine T (Hagerman and Butler, 1980a). The iodinated protein was dialyzed either against Buffer J or against Buffer J containing 15 mM DTT (dithiothreitol) prior to conducting precipitation assays. Protein compositions, isoelectric points, and molecular weights were obtained from the literature (Sugiyama et al., 1968;

Mahler and Cordes, 1971; Peters, 1975; Malmud and Drysdale, 1978; Paech, 1985).

Phlorotannin solutions (10 μg/μL) were prepared daily in methanol. Proteins were dissolved in 0.11 M sodium chloride and stored at 4°C. Most proteins were prepared as 10 μg/μL solutions, but ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC) was prepared as a saturated solution (approximately 2.5 μg/μL) and centrifuged to yield a clear solution.

Acidified acetic acid was prepared by mixing concentrated hydrochloric acid with glacial acetic acid (16 mL concentrated hydrochloric acid per 100 mL solution). Fresh solutions of DMBA (2 g/100 mL glacial acetic acid) were prepared fresh daily; this solution turned purple with age (Stern et al., 1996).

For the standard precipitation reactions at pH 5.0, Buffer J was prepared (0.1 M acetate, 0.43 M sodium chloride, pH 5.0) and stored at 4°C. For pH profiles, 0.1 M sodium phosphate buffers were prepared at various pH values between 2 and 12. A stock 1 M solution of the reducing agent DTT in water was prepared and stored at −20°C. Buffers were brought to a final concentration of 15 mM DTT daily by adding an appropriate amount of the stock solution of DTT.

Phlorotannin-Protein Precipitation Assay

We used the new DMBA assay, described more fully in Stern et al. (1996), to measure phlorotannins after they were precipitated by protein. Buffer (180 μL) was mixed with the phlorotannin solution (10 μL of 10 μg/μL phlorotannin) in conical glass centrifuge tubes (12–15 mL capacity). Protein (20 μL of 10 μg/μL protein) was added and the samples were mixed. The samples were incubated for 30 min at room temperature, and were then centrifuged at 2000 × *g* for 15 min. The supernatants were removed by aspiration and the surfaces of the pellets were gently washed with 100 μL buffer. The samples were centrifuged again for 5 min, and the supernatants removed by aspiration.

In order to quantitate the phlorotannin in the precipitates, it was necessary to redissolve the precipitated complexes. In the initial experiments with protein and phlorotannin, the protein-phlorotannin precipitates were dissolved directly in glacial acetic acid containing DMBA and hydrochloric acid. Although this was successful for preliminary experiments with *Ecklonia* tannin and bovine serum albumin, as we began to extend the work we found that in some cases the protein-phlorotannin precipitate did not completely dissolve in this reagent. We unsuccessfully tested a variety of conditions such as extremes of pH or addition of protein denaturants for dissolving the precipitates. We found that for most proteins the complex could be completely dissolved by vigorous agitation of the precipitate in 10 μL of N,N-dimethylformamide. This procedure was used in all subsequent assays.

Immediately after dissolving the pellets, 1.25 mL of the acidified acetic acid was added to each sample. It was necessary to add the acidified glacial acetic acid reagent immediately after dissolving the precipitate in the dimethylformamide to prevent destruction of the phlorotannin. At timed intervals, 1.25 mL of DMBA reagent was added to each sample; the samples were immediately mixed and placed in a water bath at 30°C. Exactly 60 min later, the absorbance of each sample was determined at 510 nm (Stern et al., 1996).

A small amount of phlorotannin adsorbed to the tubes, resulting in background absorbance. To correct for that background, a sample containing phlorotannin and buffer but no protein was treated like the other samples and its absorbance (typically about 0.06) was subtracted from the absorbance for each sample. The total amount of phlorotannin used in each experiment was determined by reacting 10 μL of the phlorotannin solution, 10 μL N,N-dimethylformamide and 1.25 mL of the acidified acetic acid with 1.25 mL of DMBA reagent for exactly 60 min at 30°C. The amount of phlorotannin precipitated was calculated as a percent of the total amount of phlorotannin.

Precipitation of ^{125}I Bovine Serum Albumin

This assay was used to determine the amount of protein precipitated by the various phlorotannins. In this assay, the amount of radiolabeled protein precipitated is determined by direct radiochemical counting. Since it is not necessary to dissolve the tannin-protein complex to count the radiolabeled protein, this assay was particularly useful for quantitating insoluble covalent complexes obtained with phlorotannins.

Tannins were dissolved in methanol and 100 μL was added to 300 μL of the appropriate buffer containing 50 μg of iodinated protein. The concentration of the tannin in the solution was set so that not all of the protein precipitated. After 15 min the samples were centrifuged, the supernatants were removed and the precipitates were gently washed with 100 μL of the appropriate buffer. The samples were centrifuged again, the supernatants were removed and the precipitates were counted with a gamma counter.

RESULTS

Precipitation of Phlorotannin by Protein

In order to determine how biologically relevant parameters affect the interaction between phlorotannins and protein, it was first necessary to optimize the conditions for precipitation. We evaluated the effects of time, temperature, and reactant concentrations on precipitation of phlorotannins by proteins. Precipitation of *Ecklonia* phlorotannins by protein occurred rapidly (Figure 2a). The

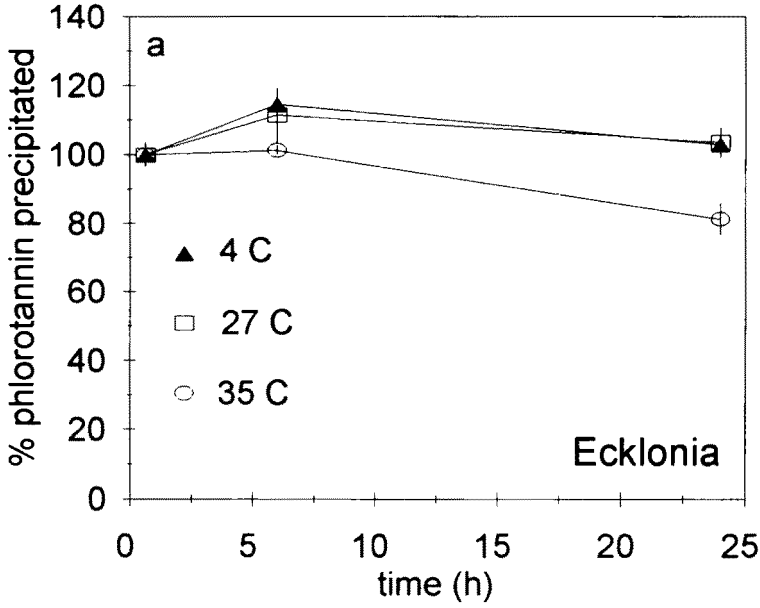
amount of phlorotannins precipitated was similar for incubation times ranging from 15 min to 24 hr, and at temperatures ranging from 4°C to 35°C. Precipitation of *Carpophyllum* phlorotannins increased slowly with time when the reducing agent DTT was present in the incubation mixture (Figure 2b). We routinely incubated phlorotannins and protein for 30 min at room temperature before analyzing the precipitate.

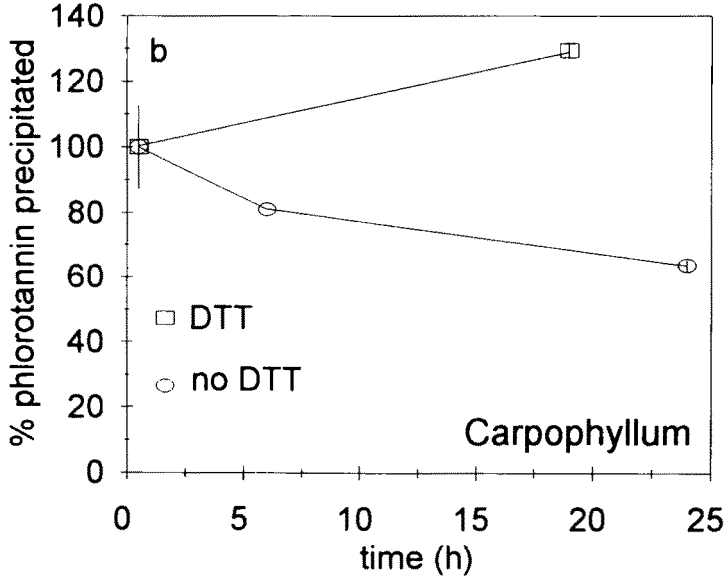
The composition of the phlorotannin-protein precipitate was dependent on both the amount of protein and phlorotannins present. When the protein-to-phlorotannin mass ratio was small ($<2:1$), between 60 and 100% of the phlorotannins were precipitated and all of the protein was precipitated. The efficiency of phlorotannin precipitation depended on the phlorotannins, the protein and the buffer used. If the protein-to-phlorotannin ratio was large ($>5:1$), less than 60% of the phlorotannins were precipitated, and only a fraction of the protein was precipitated. The mass ratio of protein-to-phlorotannin was routinely set at 2:1 to maximize the amount of phlorotannin that was precipitated.

Effects of Reducing Agents

We found that even dimethylformamide did not completely dissolve precipitates formed by *Carpophyllum* phlorotannins and certain proteins especially when the protein-to-phlorotannin ratio was small. Precipitates comprised of *Carpophyllum* phlorotannins and trypsin, chymotrypsin, or cytochrome c were dark-colored and resistant to dissolution. *Ecklonia* phlorotannins and cytochrome c also formed dark-colored precipitates which were difficult to redissolve. The color of these complexes suggested that oxidation reactions were involved, and the resistance to dissolution suggested covalent bonds had formed between the tannin and the protein. We used reducing agents to evaluate the role of oxidation. When the phlorotannins were mixed with any of these proteins in buffer containing high concentrations of reducing agents, such as 50 mM β-mercaptoethanol or 15 mM DTT, the complexes which formed were pale colored and could usually be dissolved in dimethylformamide. This indicated the importance of oxidation in phlorotannin-protein interactions, so we subsequently routinely added 15 mM DTT to the incubation buffer.

The addition of reducing agents also had the benefit of maximizing the color yield obtained in the DMBA assay. For example, when DTT was omitted from the reaction mixtures, less *Carpophyllum* phlorotannins were detected in precipitates formed during long (6–24 hr) incubations with protein than were detected after short (30 min) incubations (Figure 2b). In the presence of reducing agent, the amount of precipitated *Carpophyllum* phlorotannins increased with long incubations. It is likely that during the long incubation without reducing agent the phlorotannins spontaneously oxidized, decreasing their reactivity with DMBA. We minimized oxidative interference with the DMBA assay by incor-


a
% phlorotannin precipitated
time (h)
4 C
27 C
35 C
Ecklonia
0
20
40
60
80
100
120
140
0
5
10
15
20
25


b
% phlorotannin precipitated
time (h)
DTT
no DTT
Carpophyllum
0
20
40
60
80
100
120
140
0
5
10
15
20
25

porating the reducing agent DTT in the precipitation step and by maintaining the redissolved samples at low pH, since phenolic oxidation occurs less readily at lower pH values (Ronlan, 1978).

Composition of Phlorotannin-Protein Complexes

For all proteins tested, the amount of either *Carpophyllum* or *Ecklonia* phlorotannins detected in the precipitate was increased by the addition of the reducing agent DTT to the incubation buffer (Figure 3). Further experiments with phlorotannins from *Lobophora* indicated that the magnitude of the effect of DTT was both protein- and phlorotannin-specific. For example, precipitation of high molecular weight *Lobophora* phlorotannins by RuBPC or pepsin was not affected by DTT, but precipitation by bovine serum albumin, lysozyme, or trypsin was DTT-dependent (Figure 3). The amount of low molecular weight *Lobophora* phlorotannins precipitated was not affected by addition of DTT for any protein tested (Figure 3).

We used radiolabeled bovine serum albumin to confirm the effect of reducing agents on phlorotannin precipitation. We determined the amount of protein precipitated by phlorotannins or terrestrial tannins in the presence and absence of reducing agents. The amount of protein precipitated by *Carpophyllum* or by high molecular weight *Lobophora* phlorotannins was increased by addition of DTT to the incubation mixture (Table 1). The reducing agent did not affect the amount of protein precipitated by *Ecklonia* or low molecular weight *Lobophora* phlorotannins, or by the three terrestrial tannins. Not only was precipitation of tannic acid, a terrestrial gallotannin, insensitive to DTT (Table 2), but we found that fairly vigorous conditions were required to oxidize gallotannins. We were able to oxidize gallotannins only at pH values above 8 in the presence of an oxidant (A. E. Hagerman and S. Y. Harper-Avilla unpublished data). In contrast, we have found that some phlorotannins are susceptible to oxidation by air in slightly acidic to neutral solutions.

The activity of the reducing agent was pH-dependent (Figure 4). In general,

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FIG. 2. Kinetics of phlorotannin precipitation by bovine serum albumin. Phlorotannin (10 μg) was mixed with protein (20 μg) in pH 5 phosphate buffer. After the indicated incubations, mixtures were centrifuged, and the supernatants aspirated off. The pellets were redissolved in acidified acetic acid and reacted with DMBA in acetic acid at 30°C for exactly 60 min before the absorbance at 510 nm was determined. Each point represents the mean of three determinations and the error bars represent standard deviations. a, Precipitation of *Ecklonia* phlorotannin at 4°C, 27°C, or 35°C. b, Precipitation of *Carpophyllum* phlorotannin at 25°C in the presence or absence of DTT, a reducing agent.

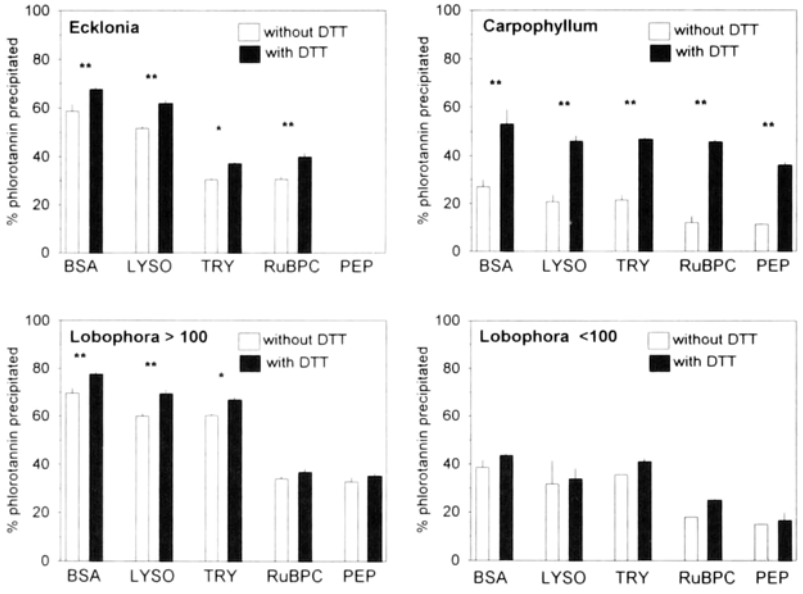


FIG. 3. Precipitation of phlorotannin in the presence and absence of DTT, a reducing agent. The standard DMBA method was used, but DTT was omitted from buffer J for reactions without DTT. Each bar represents the mean of three replicates. Significantly different values (t-test) between treatments with and without DTT are indicated (** for $P < .01$, * for $P < .05$).

addition of DTT had little effect on the composition of protein-phlorotannin complexes formed at low pH. DTT increased the amount of phlorotannin precipitated at slightly acidic, neutral, or basic pH values.

Phlorotannin-Protein Interactions

To evaluate interactions between unoxidized phlorotannins and proteins, we examined the effects of pH and of various solvents on precipitation in the presence of reducing agents. The precipitation of phlorotannins by various proteins is strongly pH-dependent (Figure 4; Figure 5). The shape of the pH profile is largely dictated by the characteristics of the protein. The pH range which favored precipitation was related to the acidity or basicity of the protein. For example, the very acidic protein pepsin (pI = 2.2) was precipitated only in acidic solutions (pH < 6). The slightly acidic proteins bovine serum albumin (pI = 4.9) and RuBPC (pI = 4.9) were precipitated in slightly acidic to neutral

TABLE 1. PRECIPITATION OF RADIOLABELED BOVINE SERUM ALBUMIN BY VARIOUS TANNINS IN THE PRESENCE OR ABSENCE OF DTT. THE AMOUNT OF ^{125}I-BOVINE SERUM ALBUMIN IN THE TANNIN-PROTEIN PRECIPITATE WAS DETERMINED BY GAMMA COUNTING. TO ENSURE THAT CHANGES IN THE AMOUNT OF PROTEIN PRECIPITATED COULD BE DETECTED, THE PROTEIN-TO-TANNIN MASS RATIO WAS SET SO THAT THERE WAS ALWAYS SOME UNPRECIPITATED PROTEIN LEFT IN THE REACTION MIXTURE. THE VALUES ARE MEAN ± SD FOR THREE REPLICATES. SIGNIFICANTLY DIFFERENT VALUES BETWEEN TREATMENTS WITH AND WITHOUT DTT ARE INDICATED (*t*-TEST; ** FOR $P < .01$, * FOR $P < .05$)

Tannin	protein: tannin mass ratio	Precipitated protein without DTT (%)	Precipitated protein with DTT (%)
Carpophyllum	12.5:1	23.6 ± 2.2	44.2 ± 0.6**
Lobophora < 100,000 amu	12.5:1	65.6 ± 5.3	77.2 ± 0.7*
Ecklonia	5:1	40.1 ± 4.1	35.7 ± 2.1
Lobophora > 100,000 amu	12.5:1	79.3 ± 1.7	79.5 ± 1.7
Sorghum	12.5:1	62.9 ± 5.4	69.0 ± 2.2
quebracho	5:1	66.9 ± 2.9	71.7 ± 1.4
tannic acid	12.5:1	92.8 ± 3.8	86.3 ± 0.4

solutions (pH 4-6). The basic proteins trypsin (pI = 10.5) and lysozyme (pI = 11.1) were precipitated in slightly acidic to basic solutions (pH 4-10).

The nature of the interaction between phlorotannin and protein was further probed by adding various organic modifiers to the reaction mixture. If tannin-protein precipitates are stabilized by hydrogen bonds between the tannin and the protein, then hydrogen bonding solvents should interfere with precipitation. If hydrophobic forces stabilize the tannin-protein complex, then nonpolar solvents or detergents should interfere with precipitation. We found that several different organic solvents influenced the precipitation of phlorotannins by bovine serum albumin. The magnitude of the effect was dependent on the properties of the solvent and on the phlorotannin (Table 2). In general, the most effective inhibitors of precipitation were dimethylformamide and dioxane. The ability of dimethylformamide to inhibit precipitation of phlorotannin by protein was consistent with its ability to dissolve phlorotannin-protein complexes. Methanol inhibited the precipitation of only a single phlorotannin, from *Ecklonia*, and stimulated precipitation of the high molecular weight phlorotannin from *Lobophora*.

The effects of the organic solvents on precipitation were concentration dependent. For example, 10% dimethylformamide or dioxane had little effect

TABLE 2. PRECIPITATION OF PHLOROTANNINS IN THE PRESENCE OF ORGANIC MODIFIERS. THE INDICATED ORGANIC SOLVENT WAS SUBSTITUTED FOR PART OF THE BUFFER TO ACHIEVE 10% SOLVENT IN THE INCUBATION MIXTURE. ALL INCUBATIONS ALSO CONTAINED 3.7% METHANOL INTRODUCED WITH THE PHLOROTANNIN. VALUES ARE THE MEAN ± SD FOR THREE DETERMINATIONS. THE DATA ARE NORMALIZED BY EXPRESSING AMOUNT OF PHLOROTANNIN PRECIPITATED AS A % OF THE AMOUNT PRECIPITATED IN THE ABSENCE OF ADDED ORGANIC SOLVENT. IN THE ABSENCE OF SOLVENT, 48.1% OF THE ADDED *Ecklonia* PHLOROTANNIN WAS PRECIPITATED; 78.4% OF THE ADDED *Carpophyllum* TANNIN WAS PRECIPITATED; 51.6% OF THE ADDED LOW MOLECULAR WEIGHT *Lobophora* TANNIN WAS PRECIPITATED; AND 59.6% OF THE HIGH MOLECULAR WEIGHT *Lobophora* TANNIN WAS PRECIPITATED

Solvent	Mode of action	*Ecklonia*	*Carpophyllum*	*Lobophora* 5-100	*Lobophora* >100
none		100	100	100	100
DMF	hydrogen bonding	3.7 ± 1.3	67.6 ± 2.5	75.7 ± 4.9	89.7 ± 1.4
acetone	hydrogen bonding	44.1 ± 2.7	87.5 ± 3.5	78.6 ± 4.9	105.4 ± 2.2
ethanol	nonpolar solvent	39.5 ± 2.2	69.2 ± 4.5	66.5 ± 6.2	121.0[a]
methanol	nonpolar solvent	79.6 ± 6.7	96.8 ± 5.8	98.7 ± 4.7	126.9 ± 6.3
dioxane	nonpolar solvent	23.6 ± 2.0	36.7 ± 4.6	85.3 ± 1.6	90.5 ± 6.0

[a] $n = 2$

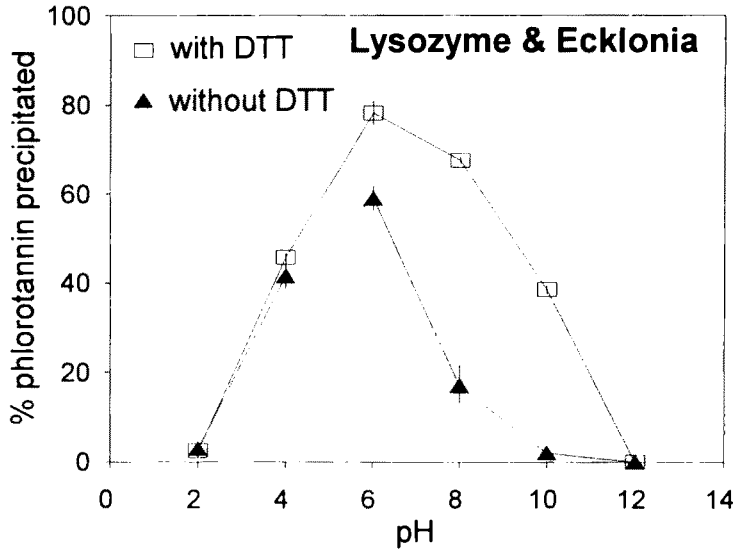


FIG. 4. Precipitation of *Ecklonia* phlorotannin by lysozyme as a function of pH and presence of DTT. Tannin and protein were incubated in 0.1 M phosphate buffers without DTT or in the same buffers containing 15 mM DTT. Points represent the means of three determinations, and the error bars represent standard deviations.

on precipitation of high molecular weight *Lobophora* phlorotannin (Table 2). However, if the mixture of phlorotannin and protein contained 20% of either of these solvents, precipitation of high molecular weight *Lobophora* phlorotannin was inhibited 60% (dioxane) or 70% (dimethylformamide).

Adding as little as 1 to 3% of the nonionic detergent Triton X 100 to incubation mixtures containing bovine serum albumin and *Ecklonia* or *Carpophyllum* phlorotannin completely prevented precipitation.

DISCUSSION

The effects of tannins or phlorotannins on herbivores varies significantly in both terrestrial (Bernays et al., 1989) and marine (Steinberg and van Altena, 1992; Steinberg et al., 1995) systems. Some of the basis for this variation is documented for terrestrial herbivores, and includes characteristics of both the herbivores and of the tannins (Martin and Martin, 1984; Bernays et al., 1989; Clausen et al., 1990; Hagerman and Robbins, 1993).

In marine systems, the effects of phlorotannins on herbivores vary as a function of the type of phlorotannin (Steinberg and van Altena, 1992; Boettcher

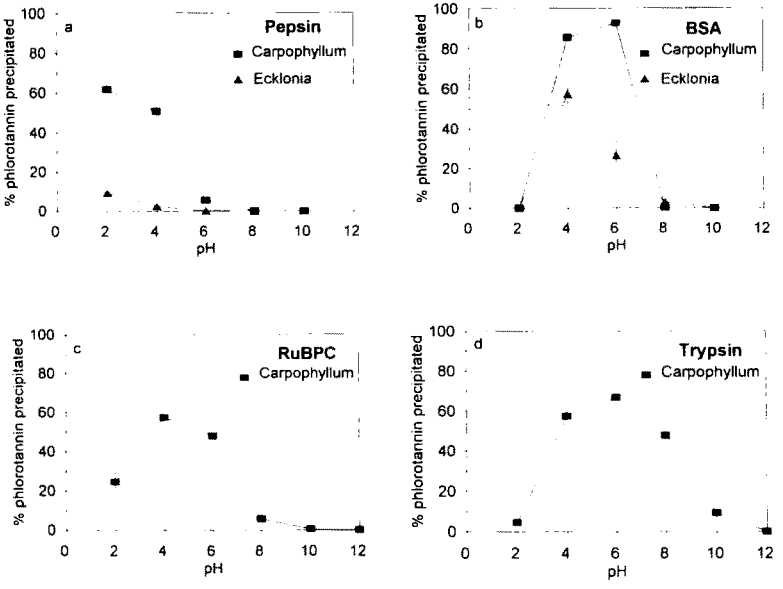


FIG. 5. Precipitation of *Carpophyllum* or *Ecklonia* phlorotannin by proteins as a function of pH. Tannin and protein were incubated in 0.1 M phosphate buffers containing 15 mM DTT. Points represent the means of three determinations, and the error bars represent the standard deviations. a, Pepsin. b, Bovine serum albumin. c, RuBPC. d, Trypsin.

and Targett, 1993), the characteristics of the gut environment (Tugwell and Branch, 1992), the herbivore species (Steinberg and van Altena, 1992) and the geography (Van Alstyne and Paul, 1990; Steinberg and van Altena, 1992; Steinberg et al., 1995). For the most part, the specific mechanisms of action of phlorotannins which underlie this variation are unknown.

Reactions of Oxidized Phlorotannins

Appel (1993) proposed that phenolic oxidation may have significant ecological implication and we have obtained detailed experimental evidence for reactions between oxidized tannins and protein. A mechanism for formation of covalent complexes between protein and oxidized plant phenols was proposed by Pierpoint (1969a), and reactions between oxidized simple phenolics, such as chlorogenic acid, and protein have been demonstrated (Pierpoint, 1969b; Felton et al., 1992). Leatham et al. (1980) demonstrated that tannic acid treated with peroxidase and hydrogen peroxide precipitated more protein than untreated tannic acid. However, evidence accumulated under a wide range of conditions indicates

that in the absence of added oxidants, oxidation is not involved in the reactions between terrestrial tannins and proteins (Haslam, 1989; Hagerman, 1992). In contrast, we have found that the phlorotannins from marine algae spontaneously oxidize and react with protein to form dark colored complexes resistant to dissolution unless a reducing agent is present.

We initially recognized oxidation in the phlorotannin system because of difficulties we encountered when attempting to redissolve some phlorotannin-protein precipitates for analysis. Dark-colored precipitates resistant to solubilization were obtained especially when *Carpophyllum* phlorotannins were incubated with protein in alkaline solution. The precipitates could not be redissolved by hydrogen bond-breaking solvents, such as dimethylformamide or by protein denaturants, such as sodium dodecyl sulfate, leading us to postulate that covalent bonds were stabilizing these complexes. The color of the precipitates was consistent with the orange-brown colors of oxidized phenolics (Pierpoint, 1969a).

We used mild reducing agents to demonstrate the importance of oxidation in phlorotannin-protein interactions. We found that when 15 mM DTT was included in the mixture of phlorotannins and protein, light colored precipitates formed. These precipitates were easily dissolved in dimethylformamide, suggesting that the protein-phlorotannin complex formed under these conditions involved noncovalent interactions. The reducing agents were effective with a variety of proteins and phlorotannins over a broad pH range. We obtained similar results using an alternative reducing agent, β-mercaptoethanol, in place of DTT.

The method of analysis that we employed (Stern et al., 1996) is particularly well suited to detecting insoluble covalent complexes between oxidized tannins and protein. Our method is dependent on resolubilizing the precipitate; irreversible, covalent complexes were readily recognized. Tugwell and Branch (1992) examined phlorotannin-protein interactions but did not report any effects that can be attributed to oxidation. They analyzed the supernatant after removal of the precipitate and thus did not assess solubility or other characteristics of the precipitates. Our study was not designed to examine soluble tannin-protein complexes stabilized by either noncovalent or covalent bonds (Hagerman and Robbins, 1987).

In most of our experiments, more phlorotannins were detected in precipitates formed under reducing conditions than in precipitates formed under oxidizing conditions (Figure 2). This increased precipitation could be an artifact resulting from the higher color yield of DMBA with reduced phlorotannins. An additional source of artifact could be the improved solubility of complexes formed under reducing conditions, since the assay only detects the phlorotannins that can be redissolved after precipitation. To eliminate these possible interferences, we performed precipitation reactions using a radiolabeled protein. Precipitated, labeled protein is determined by direct radiochemical counting so interferences in the assay are eliminated. For *Carpophyllum* phlorotannins and moderate

molecular weight *Lobophora* phlorotannins more protein was precipitated under reducing conditions than under oxidizing conditions (Table 1). This clearly demonstrates that the effects of reducing agents on phlorotannin precipitation are not artifacts of our method of analysis.

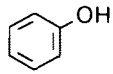
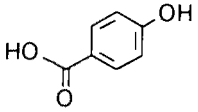
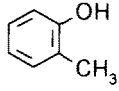
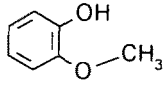
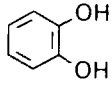
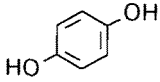
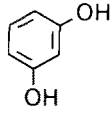
Oxidation is more important for interactions of phlorotannins with some proteins and less important for other proteins. The proteins examined differ in many respects, including size, amino acid composition and isoelectric point. The role of oxidation for precipitation of phlorotannins by RuBPC was particularly variable. RuBPC precipitation of *Carpophyllum* phlorotannins was enhanced more by the addition of DTT than was the precipitation of any other protein, but addition of DTT had no effect on precipitation of high molecular weight *Lobophora* phlorotannins by RuBPC. RuBPC is unusually large (560,000 amu) and is a 16-mer comprised of two types of subunits. The other proteins used in these experiments range in size from 14,400 amu (lysozyme) to 65,000 amu (bovine serum albumin) and are all single polypeptide chains. RuBPC contains an unusual number of reduced thiols (96 cysteines per 16-mer of spinach RuBPC), which may make it particularly susceptible to oxidation. Of the other proteins examined, bovine serum albumin contains 17 disulfide bonds (cystines) and one cysteine residue; the other proteins contain three to six cystines and no cysteine.

Tannin Structure and Oxidation Reactions

The tendency of phenolics to oxidize is affected by the nature of substituents and the position of substituents on the phenolic (Ronlan, 1978). The presence of an electron withdrawing group such as a carboxylic acid or an ester makes the phenolic more difficult to oxidize; thus, the redox potential of *para*-hydroxy benzoic acid is higher than the redox potential of phenol (Table 3). Electron donating groups such as alkyl, aryl, ether, or hydroxyl groups make the phenolic easier to oxidize. Among the electron donating groups, alkyl and aryl groups have the smallest effect, ether groups have a larger effect and hydroxyl groups have the largest effect on oxidation potential (compare *ortho*-methyl phenol, *ortho*-methoxyphenol and catechol, Table 3). Substituents *ortho* or *para* to a phenolic group change the redox potential more than *meta* substituents. For example, the redox potentials of catechol and hydroquinone (*ortho* and *para* substituted, respectively) are much lower than the redox potential of phenol while the redox potential of resorcinol (*meta* substituted) is intermediate (Table 3).

We found that the susceptibility of tannins to oxidation can be explained by examining the structural characteristics of the tannins. The susceptibility of some phlorotannins to oxidation is a consequence of extensive substitution by aryl ethers (1, 2, and 3, Figure 1). The ether groups are strong electron donating

TABLE 3. REDOX POTENTIALS OF SIMPLE PHENOLICS

Structure	Substitutent pattern and type	Redox potential (V)
	phenol unsubstituted	0.62[a] 0.63[b]
	*para*-hydroxybenzoic acid carboxylic acid electron withdrawing	0.72[b]
	*ortho*-methyl phenol alkyl electron donating	0.56[b]
	*ortho*-methoxy phenol ether electron donating	0.46[b]
	catechol *ortho* hydroxyl electron donating	0.18[a]
	*para*-hydroquinone *para* hydroxyl electron donating	0.18[a]
	resorcinol *meta* hydroxyl electron donating	0.39[a]

[a] $E_{p/2}$ vs. standard calomel electrode, pH 7 phosphate (Meites & Zuman 1974)
[b] $E_{1/2}$ vs. standard calomel electrode, pH 5.6 isopropanol/H_2O (Suatoni et al. 1961)

groups, especially when they are positioned *ortho* or *meta* to phenolic groups as in the phlorotannins. Ether substituents facilitate oxidation of the resorcinol-like phenolic subunits of the phlorotannins.

The phlorotannins from *Carpophyllum* were more susceptible to oxidation than any other tannin we examined. Both the amount of protein and the amount of phlorotannins precipitated were enhanced by the addition of reducing agent to the protein-*Carpophyllum* mixtures (Figure 3, Table 1). The mixture of phlorotannins found in *Carpophyllum* includes fuhalols (1, Figure 1) (Ragan and Glombitza, 1986) and hydroxyphlorethols (2, Figure 1) (Glombitza and Li, 1991a). Both of these types of phlorotannin characteristically have *ortho* hydroxy groups on the terminal unit. The addition of the strong electron donating hydroxyl group *ortho* to the other hydroxyl groups on the terminal unit of the *Carpophyllum* phlorotannins enhances sensitivity to oxidation. In contrast, the phlorotannins from *Ecklonia* are less susceptible to oxidation (Figure 2, Table 1); phlorotannins from *Ecklonia* such as phlorethols (3, Figure 1) do not contain any hydroxyl groups in the *ortho* orientation (Ragan and Glombitza, 1986). We found that the phlorotannins from *Lobophora*, like those from *Ecklonia*, have a limited tendency to oxidize, but nothing is known about the structural chemistry of the phlorotannins from *Lobophora*.

These results suggest that structural differences between the phlorotannins may substantially influence the interaction between phlorotannins and proteins. Consequently, the ability of herbivores to counteract the effects of different phlorotannins by controlling the redox potential of the gut will also vary. Based on the limited structural data available, we predict that ether-linked phlorotannins which have *ortho* substituted hydroxyl grops on one or more rings will generally be the most susceptible to oxidation. We predict that phlorotannins which are aryl-aryl linked, such as the fucols (4, Figure 1), will be least susceptible to oxidation, since aryl groups are weaker electron donating groups than aryl ethers. This prediction could be tested by using methods like those developed by Glombitza (e.g., Glombitza and Li, 1991a; Glombitza and Li, 1991b; Li and Glombitza, 1991) to determine the structures of isolated phlorotannins, and then determining the redox potentials of those purified compounds. Our studies were done with mixtures of phlorotannins, making it impossible to specifically evaluate the contribution of each type of phlorotannin to oxidation reactions.

The terrestrial tannins did not appear to oxidize spontaneously (Table 2) despite the catechol-like arrangement of hydroxyl groups common to these tannins (5 and 6, Figure 1). Examination of the other substituents on the terrestrial tannins explains their insensitivity to oxidation. For the gallotannins (5, Figure 1), each of the phenolic rings bears at least one electron withdrawing ester group, diminishing susceptibility to oxidation. The condensed tannins have alkyl substituents on both the phenolic rings (A ring and B ring of 6, Figure 1). Alkyl substituents are poor electron donating groups, and apparently do not enhance

oxidizability of the phenolic rings sufficiently to promote air oxidation of the condensed tannins. It would be useful to directly determine the redox potentials of various phlorotannins and terrestrial tannins in order to better understand their relative susceptibility to oxidation.

Noncovalent Phlorotannin-Protein Interactions

Insight into the types of bonds that stabilize tannin-protein complexes can be obtained by varying the composition of the solution in which tannin-protein complexes are formed. For example, covalent bonds between oxidized phenolics and protein are likely to form at high pH since phenolics are more readily oxidized at high pH (Ronlan, 1978). Our observations on covalent complexes were consistent with that general trend. In the absence of reducing agent, we detected brown complexes resistant to dissolution at pH values above 4 but not at more acidic pH values.

The pH profiles we obtained for precipitation of reduced phlorotannins by proteins are similar to the profiles for precipitation of terrestrial tannins (Hagerman and Butler, 1978; Martin and Martin, 1983; Hagerman and Klucher, 1986) or phlorotannins (Tugwell and Branch, 1992) for the same proteins. Precipitation of proteins generally occurs most readily at pH values near the pI, because ionic repulsion is minimized at that pH (Mahler and Cordes, 1971). The general pattern of precipitation by phlorotannins was consistent with that trend.

High pH should favor either ionic or covalent interaction; at pH values above about 9 phenolic groups are ionized. Reduced phlorotannins were not precipitated by most proteins at high pH values. However, trypsin and lysozyme precipitate some phlorotannin at pH 10 (Figure 3, Figure 4), suggesting either ionic interactions or oxidation of the phlorotannin even in the presence of DTT. Further studies are necessary to distinguish between these two possibilities.

In the presence of the reducing agent, phlorotannin-protein interactions were inhibited by a variety of solvents (Table 2). Inhibition by hydrogen-bonding solvents such as dimethylformamide or acetone provides evidence that hydrogen bonding plays an important role in precipitation. Dimethylformamide is a more effective inhibitor because it is a stronger hydrogen bond acceptor than is acetone.

Hydrophobic forces appear to play a minor role in formation of phlorotannin-protein precipitates. The temperature independence of precipitation (Figure 1) suggests that hydrophobic forces are not important in formation of phlorotannin-protein complexes (Kennedy, 1990). Furthermore, the dependence of precipitation on pH suggests that hydrophobic forces do not stabilize these complexes, since hydrophobic interactions are generally pH-independent. Nonetheless, precipitation is inhibited by some nonpolar solvents such as dioxane, and ethanol inhibited precipitation more effectively than its more polar analogue,

methanol (Table 2). Tugwell and Branch (1992) found that gut surfactants inhibited the interaction between the *Fucus* phlorotannin and RuBPC and we found that the nonionic detergent Triton X 100 inhibited precipitation. The effects of these nonpolar solution modifiers indicates that the interaction between reduced phlorotannins and proteins involves nonpolar interactions as well as strong hydrogen bonds.

CONCLUSIONS

This is the first study to compare the reactions of phlorotannins and terrestrial tannins with proteins. The work demonstrated that oxidized tannins can form covalent complexes with proteins, and confirmed the hypothesis that redox conditions may be an important determinant of the biological activity and fate of tannins (Appel, 1993). Our study suggests that oxidative reactions are common among certain phlorotannins, but that oxidation of terrestrial tannins may be rare. It is logical to conclude that gut redox conditions should be examined in marine herbivores which consume phlorotannin-containing brown algae. For example, the redox potential of the gut might be relatively unimportant for an herbivore which consumes *Ecklonia*, but gut redox potential could be an important determinant of the fate and effects of *Carpophyllum* phlorotannin.

In addition to redox conditions, we found that pH and solution composition influenced phlorotannin-protein interactions. The phlorotannin-protein complexes which formed in a reducing environment were similar to terrestrial tannin-protein complexes that have been characterized earlier. Thus, we believe that in marine herbivores, as in terrestrial herbivores, variation in sensitivity to dietary tannins is due to variation in herbivore gut chemistry such as pH, surfactant concentrations, and presence of tannin-binding proteins (Bernays et al., 1989; McArthur et al., 1991). We have recently confirmed that concentrations of metal ions (sodium, potassium, calcium, and magnesium) significantly influence precipitation of phlorotannins by proteins (Tugwell and Branch, 1992; Mason and Hagerman unpublished data). These results suggest that marine herbivores could control the effects of tannins by controlling salt concentration in the gut. We are currently extending those studies to explore the basis for the metal ion effects.

We have demonstrated that one of the activities of phlorotannins, ability to precipitate proteins, is related to the chemical structures of the tannins. Similar structure-activity relationships have been found for terrestrial tannins. Understanding how structure influences biological activity is an essential component to understanding the effects of phlorotannins on herbivores.

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REASSESSMENT OF THE ROLES OF THE PERITROPHIC ENVELOPE AND HYDROLYSIS IN PROTECTING POLYPHAGOUS GRASSHOPPERS FROM INGESTED HYDROLYZABLE TANNINS

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Abstract—We examined several of the mechanisms that have been reported to enable polyphagous grasshoppers (Orthoptera: Acrididae) to tolerate ingested hydrolyzable tannins: hydrolysis, adsorption on the peritrophic envelope, and peritrophic envelope impermeability. None of these mechanisms explain the tolerance of *Melanoplus sanguinipes* to ingested tannic acid. In this species, tannin hydrolysis was 12–47% complete, adsorption accounted for less than 1% of the tannic acid contained in the midgut, and the peritrophic envelope was permeated by several gallotannins. The foregut is the main site for the chemical transformation of tannic acid in this species. In *Phoetaliotes nebrascensis*, hydrolysis was more extensive (82% complete), but the peritrophic envelope was readily permeated by two gallotannins. Oxidizing redox conditions were found in the guts of both species, and ingested tannins were oxidized in *M. sanguinipes*. We hypothesize that the tolerance of some polyphagous grasshoppers to ingested hydrolyzable tannins may be the consequence of their ability to tolerate the reactive oxygen species generated by polyphenol oxidation, whereas others may rely on rapid and extensive hydrolysis.

Key Words—Orthoptera, Acrididae, grasshopper, *Melanoplus sanguinipes*, *Phoetaliotes nebrascensis*, peritrophic envelope, peritrophic membrane, hydrolyzable tannin, tannic acid, oxidation, hydrolysis.

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INTRODUCTION

Grasshoppers (Orthoptera: Acrididae) vary greatly in their sensitivity to ingested hydrolyzable tannins. Polyphagous species are remarkably tolerant, being able to survive on diets containing 20% tannic acid without inhibition of feeding, reduction in the efficiency of utilization of nutrients, formation of lesions in the midgut epithelium, or any other adverse effect commonly associated with tannin sensitivity (Bernays, 1978; Bernays and Chamberlain, 1980; Bernays et al., 1980). Graminivorous species are less tolerant of tannic acid than polyphagous species. For several graminivorous grasshoppers, tannic acid has been shown to reduce feeding and cause lesions in the midgut, which may prove lethal if the feeding period is sufficiently long (Bernays, 1978; Bernays et al., 1980; Raubenheimer, 1992). Two mechanisms have been proposed to contribute to the tolerance of polyphagous grasshoppers to tannic acid: rapid hydrolysis (Bernays, 1978; Bernays and Chamberlain, 1980) and containment within the peritrophic envelope (Bernays et al., 1980; Bernays and Chamberlain, 1980).

Since the publication of the seminal papers of Bernays and others (Bernays, 1978; Bernays and Chamberlain, 1980; Bernays et al., 1980), there have been a number of advances in our understanding of the effects of tannins on insects and of the permeability properties of the peritrophic envelope, advances suggesting that the explanation proposed for tannin tolerance in generalist grasshoppers is still not complete. For example, our recent studies of tolerance in Lepidoptera (Barbehenn and Martin, 1992, 1994), which demonstrated that the containment of tannic acid within the endoperitrophic space is not sufficient to confer tannin tolerance, requires a re-examination of the putative protective role of the peritrophic envelope in grasshoppers. In addition, the recognition that the agents responsible for the adverse effects of ingested polyphenols are often not the polyphenols themselves, but rather oxidation products, such as quinones, or by-products of oxidation, including reactive oxygen species such as hydrogen peroxide, superoxide anion radicals, and hydroxyl radicals (Appel, 1993; Ahmad, 1995; Cadenas, 1995; Felton, 1995; Summers and Felton, 1994), requires a re-examination of the chemical fate of ingested polyphenols in grasshoppers in order to determine whether or not they are oxidized.

The purpose of the present study is to re-examine the basis for tannin tolerance in polyphagous grasshoppers. We have examined several potential mechanisms in two grasshopper species in the subfamily Melanoplinae, *Melanoplus sanguinipes* and *Phoetaliotes nebrascensis*. *M. sanguinipes* is a highly polyphagous species with a diet varying from 50–78% forbs over its range in the midwestern United States (Gangwere et al., 1976; Joern, 1983). Although *P. nebrascensis* consumes more grasses and fewer forbs than *M. sanguinipes* (Joern, 1983), it has mixed-feeder mandibles (Isely, 1944) indicative of a polyphagous species. *M. sanguinipes* has a high tolerance to ingested tannins (Ber-

nays et al., 1980). We are unaware of any studies to determine the sensitivity of *P. nebrascensis* to ingested tannins. However, tannic acid is not an effective feeding deterrent for either species (Bernays et al., 1980; Mole and Joern, 1994).

In our experiments we used commercial tannic acid from oak galls so that we could compare our results with those of earlier workers. However, since the simple gallotannins comprising tannic acid differ from the more complex gallotannins found in the common host plants of polyphagous grasshoppers, we have also included in our experiments a tannin isolated from fireweed (*Epilobium angustifolium*), oenothein B. This tannin is representative of a widely occurring class of oligomeric hydrolyzable tannins that consist of core carbohydrates linked by oxidatively coupled galloyl groups (Okuda et al., 1993).

In this study, we have conducted experiments that address the following questions: (1) How extensively are hydrolyzable tannins hydrolyzed while passing through the gut? (2) In what region of the gut does most of the hydrolysis occur? (3) Do redox conditions in the gut favor polyphenol oxidation? (4) Are ingested tannins oxidized in the gut? (5) How does the fate of a complex hydrolyzable tannin (fireweed tannin) differ from the fate of the simple gallotannins found in tannic acid? (6) How much tannic acid is adsorbed by the peritrophic envelope? (7) Is the peritrophic envelope permeable to tannic acid?

METHODS AND MATERIALS

Insects and Plants. Eggs of *M. sanguinipes* were obtained from the USDA (Bozeman, MT). A colony was maintained in a screen cage (1 m × 0.5 m × 0.5 m) with a 150-W light bulb placed against the screen to provide a heat gradient (16h L:8h D). Nighttime temperature was ambient (ca. 22°C). Insects were reared from egg-hatch on romaine lettuce (*Lactuca sativa longifolia*) and wheat bran. *P. nebrascensis* were collected as third- and fourth-instar nymphs in western Nebraska. A colony was maintained on rye (*Secale cereale*) seedlings and bran under the same conditions described for *M. sanguinipes*. Adult insects were used for all experiments.

Polyphenols. Tannic acid from *Quercus infectoria* (Fagaceae) galls was purchased from Sigma Chemical Company (St. Louis, MO; lot 64F-0049). Fireweed tannin was extracted from *E. angustifolium* (Onagraceae) leaves (Zhao, 1995). Gallic acid was purchased from Sigma Chemical Co.

Polyphenol Analysis. The gallotannins present in tannic acid were identified by comparing chromatograms (normal- and reverse-phase HPLC) with chromatogams of authentic samples of galloyl glucose esters isolated from tannic acid on a Sephadex LH-20 column (Hagerman et al., 1992). Calibration curves (plotting log retention time as a function of the number of galloyl groups) were constructed using data obtained with authentic standards in either normal- or

reverse-phase HPLC. The regression equations are log retention time = (0.085 × no. galloyl groups) + 0.415 (r^2 = 0.999) for normal-phase, using the linear portion of the curve up to pentagalloyl glucose (Figure 1), and log retention time = 0.160 × no. galloyl groups) + 0.098 (r^2 = 0.998) for reverse-phase, using the linear portion of the curve between tri- and pentagalloyl glucose. Purified fireweed tannin is composed primarily of oenothein B (Okuda et al., 1993), as determined by NMR and mass spectrometry (Zhao, 1995).

Quantitative analyses of polyphenols in sample extracts were conducted with reverse-phase HPLC using a Vydac C-18 column (4.5 mm × 250 mm; 5 μ) and Supelco C-18 guard column (10 μ). The mobile phase was 23% (v/v) acetonitrile in double-distilled water containing 1% acetic acid, unless otherwise noted. The flow rate of the mobile phase was 1.0 ml/min. Sample aliquots (20–30 μl) were injected with a Shimadzu autoinjector. Peaks were detected at 280 nm (Shimadzu UV-visible detector, 0.002 AUFS) and quantified with a Shimadzu C-R4A integrator. In all cases, peak areas were corrected for the presence of interfering substances found in the control samples. Standard curves were calculated for tannic acid, fireweed tannin and gallic acid to convert peak areas to μg polyphenol.

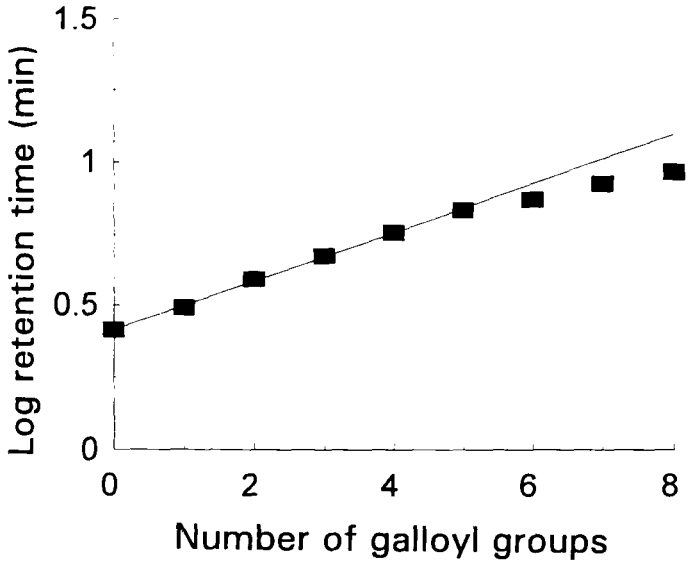

FIG. 1. Normal-phase HPLC calibration curve for gallic acid and galloyl glucose esters. The linear portion of the curve fits the regression equation log retention time = (0.085 × no. galloyl groups) + 0.415 (r^2 = 0.999).

Polyphenol Budgets. Dry-mass polyphenol budgets were determined using insects fed either artificial diet or foliage. Polyphenol-containing artificial diet was prepared by mixing the polyphenol (2.6% DW tannic acid, 2.6% DW fireweed tannin, or 2.2% gallic acid) with the dry ingredients of the Douglas fir tussock moth diet (Bioserve; Frenchtown, NJ), and hydrating as directed by the manufacturer. Polyphenol-coated lettuce (2.5% DW tannic acid or 4.6% DW gallic acid) and rye grass (4.0% DW tannic acid or 4.2% DW gallic acid) were prepared by allowing 15 μl or 13 μl of a 70% acetone solution of the polyphenol to evaporate on 22 mm diameter lettuce discs or 8 cm long rye grass leaves. Control lettuce discs and grass leaves were treated with 15 μl and 13 μl of 70% acetone, respectively.

Insects were placed individually in ventilated 470 ml plastic containers in an incubator (30°C; 16:8 light:dark) and randomly assigned to treated or control food. Initial fresh weights of food offered to each insect, and fresh weights of representative samples of each food, were measured to the nearest 0.1 mg. After feeding for one day they were fed a polyphenol-free chaser diet (foliage for insects previously fed artificial diet, and artificial diet for insects previously fed foliage). Frass was collected once approximately 24 hr after the initiation of feeding on the treated or control diets, and again after chaser diet appeared in the frass. All frass pellets were examined under a dissecting microscope, so that pellets containing egested polyphenols (i.e., pellets containing the treated diet) could be separated from pellets consisting entirely of the chaser diet. To confirm that all egested polyphenols were contained in the frass from the treated food, samples of frass from the chaser diets in three experiments were also analyzed for polyphenols. No tannic acid was found in frass from the chaser diets, but 1–4% of the total gallic acid egested was contained in this frass. Frass samples were stored at −20°C until all samples were collected, and lyophilized. Food samples for determining %DW, and uneaten food remains, were dried at 60°C (2 d). The amount of food consumed (DW) was calculated as the difference between food offered and food remaining.

Polyphenols were quantified in ground lyophilized frass and food samples. Entire samples or 5 mg aliquots were extracted three times in 1.0 ml of 70% acetone containing 0.001 M ascorbic acid at 22°C (±1°C). Extracts were centrifuged (4 min, 13,600 g), and the supernatant solutions were pooled, concentrated under a stream of nitrogen, and lyophilized. Lyophilized extracts were resolubilized in 500 μl of mobile phase (20 or 23% acetonitrile in double-distilled water containing 1% acetic acid), filtered (0.45 μ, Gelman GHP), and analyzed as described above. In most experiments using artificial diet, the gallotannins that eluted in the range of 4–8 min (Figure 2) were quantified. The gallotannins that eluted in the range of 5–13 min (Figure 2) were quantified for budgets using lettuce or rye grass. The water content of each polyphenol used was measured after drying at 60°C and subtracted from the weights of standards

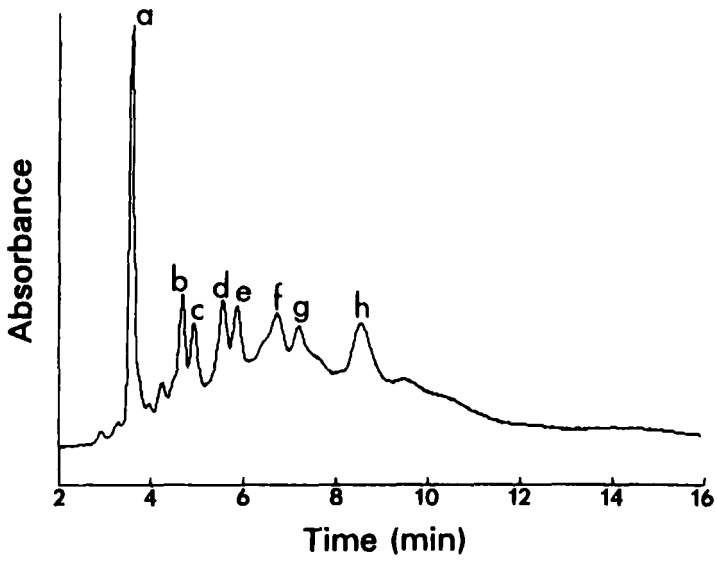

FIG. 2. Tannic acid separated on a Vydac reverse phase (C-18) column using 23% aqueous acetonitrile (with 1% acetic acid) as the mobile phase. Peak a is gallic acid, b and c are isomers of trigalloyl glucose, d and e are isomers of tetragalloyl glucose, f and g are isomers of pentagalloyl glucose, and h is hexagalloyl glucose.

and amounts of polyphenols added to food. Tannic acid is stable in the artificial diet that we used, and can be completely recovered using our extraction and HPLC methods (Barbehenn and Martin, 1992).

Tannin Hydrolysis. The extent of tannin hydrolysis was calculated from measurements of the amounts of gallic acid (GA) ingested and egested by insects used to measure tannin budgets. The extent of hydrolysis of galloyl esters in the ingested tannin was calculated from the expression 100 × (μg GA in frass derived from hydrolysis)/(theoretical μg GA yielded by complete hydrolysis of ingested tannin). The amount of gallic acid in frass derived from hydrolysis was calculated as (μg GA egested) − [(μg free GA ingested) × (% recovery in frass of ingested GA)]. The theoretical amount of gallic acid yielded by complete hydrolysis of ingested tannin was calculated from the expression (μg tannin ingested) × (μg GA produced per μg tannin hydrolyzed) × (% recovery in frass of ingested GA). The weights of gallic acid and tannin ingested were calculated from the expressions (mg food consumed) × (% GA in food) and (mg food consumed) × (% tannin in food), respectively. Tannic acid was found to yield 70% of its weight in gallic acid upon complete hydrolysis (Barbehenn and Martin, 1992). Based upon this determination, we calculated that tannic

acid would yield 69% gallic acid if the free gallic acid was removed from the tannic acid before hydrolysis. We used the calculated yield of 69% gallic acid in this study. Using the structural formula for fireweed tannin (Okuda et al., 1993), it can be calculated that this tannin would yield 22% of its weight in gallic acid upon complete hydrolysis. The percent recovery in frass of ingested gallic acid was found to be 81.5% in artificial diet and 79.8% on lettuce for *M. sanguinipes*, while 100% of ingested gallic acid was recovered in the frass of *P. nebrascensis*.

Changes in Tannic Acid and Gallic Acid Concentrations Along the Gut. *M. sanguinipes* were fed an artificial diet containing 2.6% tannic acid. Insects were dissected, as described above, and samples (2–20 mg fresh weight) of the foregut, anterior midgut, posterior midgut, and hindgut contents were quickly removed and weighed to the nearest 0.01 mg on a Cahn microbalance. Samples were immediately placed in test tubes containing 1.0 ml of 70% acetone containing 0.001 M ascorbic acid, macerated, extracted, and stored covered with parafilm at −20°C (4 d). Extracts were centrifuged, and supernatant solutions were evaporated under a stream of nitrogen and lyophilized. Lyophilized extracts were resolubilized in 500 μl of mobile phase and analyzed using HPLC as described above (30 μl aliquots). Polyphenols that eluted between 4.5–10 min (Figure 2) were quantified in standards and extracts. Concentrations of polyphenols were expressed on a dry weight basis by determining the average percent dry weight (60°C, 2d) of the gut contents in each of the regions of the gut from three insects.

The concentrations of tannic acid and gallic acid that would have been present had there been no assimilation of nutrients from the gut were calculated from the actual concentrations measured: 100 × (μg polyphenol)/[(μg sample) × (1 − assimilation efficiency)]. The assimilation efficiency (55.9% ±4.3 SE) was measured in *M. sanguinipes* using food and other conditions identical to those used in this experiment. The assumption underlying this calculation is that an assimilation efficiency of 55.9% approximates the actual assimilation efficiency throughout the midgut (Dadd, 1970; House, 1974). No correction of the polyphenol concentrations measured in the foregut was necessary since absorption from the foregut is negligible in grasshoppers (Maddrell and Gardiner, 1980).

Gut pH and Redox Conditions. Insects that had fed continuously on their rearing plants were chilled and dissected as described above. Microneedle pH (MI-408P) and silver-silver chloride reference (MI-401) microelectrodes (Microelectrodes, Londonderry, NH) were inserted through pairs of small holes cut in the gut wall. Redox potentials were measured with 0.02 in. platinum redox (MI-800) and silver-silver chloride reference (MI-401) microelectrodes using the same holes immediately following the pH measurements. Measurements in *M. sanguinipes* were made with a Metrohm/Brinkman (model 103)

millivolt and pH meter, and those in *P. nebrascensis* were made with a Corning (model 340) millivolt and pH meter. Observed redox potentials were converted to standard redox potentials (E_h) by adding 200 mV, and pe was calculated as $E_h/59.2$ (Appel and Martin, 1990). Overall redox conditions were summarized by the "redox parameter" (pH + pe), which gives equal weight to proton and electron availability in determining the thermodynamic favorability of oxidation in a complex system (Appel and Martin, 1990).

Polyphenol Oxidation. The amounts of soluble brown pigments in the food and frass of control and polyphenol-fed grasshoppers were measured as an indicator of the oxidation of ingested polyphenols (Barbehenn and Martin, 1994). Aliquots (5 mg) were extracted three times in 1.0 ml of 70% acetone containing 0.001 M ascorbic acid at 22°C (±1°C). Extracts were centrifuged (4 min, 13,600 g). The supernatant solutions were pooled, and the absorbance (420 nm) of each solution was measured with a Zeiss spectrophotometer. The net amount of brown pigment formed per mg frass was calculated by subtracting the amount of brown pigment present per mg in the food. Brown pigment formation in the frass of insects fed food with or without added polyphenols was compared with Mann-Whitney U-tests using SYSTAT (Wilkinson, 1990).

Adsorption of Tannic Acid on the Peritrophic Envelope. Tannic acid adsorption *in vivo* was assayed by feeding *M. sanguinipes* tannic acid-coated lettuce leaf discs (3.3% tannic acid). Leaf discs were coated with 15 μl of a solution of tannic acid (27 mg/ml) in 70% acetone (treatment) or 70% acetone (control). After the acetone had evaporated, two leaf discs were fed to each insect. Freshly treated leaf discs were offered after 12 hr. The insects were kept in ventilated plastic containers in an incubator at 30°C (16 hr L: 8hr D). After approximately 24 hr, chilled (−20°C, 12 min) insects were dissected, and the peritrophic envelope surrounding the food in the midgut and anterior hindgut, but not the caeca, was removed as one piece from the gut. Although the peritrophic envelope present in the caeca was not extracted, the peritrophic envelope in the main region of the midgut includes peritrophic envelope that had previously been in the caeca. The peritrophic envelope was cut longitudinally, separated from the food contents, and rinsed for 15–30 sec in three 100 μl drops of a buffer (pH 7.0) containing 5 mM Tris(hydroxymethyl)aminomethane, 45 mM Tris(hydroxymethyl)aminomethane hydrochloride, 20 mM $CaCl_2$, 3 mM NaCl, 227 mM KCl, 41 mM $MgSO_4$, 6 mM ascorbic acid, and 440 mM fructose. The peritrophic envelope was drained for 10 sec on filter paper and weighed to the nearest 0.01 mg. Polyphenols adsorbed on the peritrophic envelope were extracted twice at 22°C in 500 μl of 70% acetone containing 0.001 M ascorbic acid. Extracts were centrifuged (4 min, 13,600g) and the pooled supernatant solutions were concentrated under a stream of nitrogen after brief storage (12 h, −20°C). Lyophilized extracts were resolubilized in 250 μl of mobile phase, filtered, and 30 μl aliquots were analyzed with HPLC as described above. Fresh

weights and dry weights (60°C, 2 d) of the peritrophic envelope of six control insects were measured to the nearest 0.01 mg in order to express the amount of polyphenols adsorbed on the peritrophic envelope on a dry weight basis. The dry weight/wet weight ratio of the peritrophic envelopes was 0.19 ± 0.09. The gut contents of the dissected insects were also dried and weighed to calculate the maximum amount of tannic acid that was present in the endoperitrophic space.

To confirm that tannic acid was completely removed from the peritrophic envelopes by extraction in 70% acetone, each peritrophic envelope was hydrolyzed in 500 μl of 2 M HCl at 100°C for 20 min. Hydrolysates were neutralized with 500 μl of 2 M NaOH and lyophilized. The lyophilized hydrolysates were resolubilized in mobile phase (300 μl), filtered, and 30 μl aliquots were analyzed with HPLC, as described above. No gallic acid was observed in the chromatograms of the hydrolysates, demonstrating that tannic acid had been completely extracted from the peritrophic envelopes in 70% acetone.

Permeability of the Peritrophic Envelope to Polyphenols. M. sanguinipes that had fed on lettuce were chilled (−20°C, 9 min) and their guts were excised. The foregut and anterior midgut (including the caeca) were removed. The hindgut was ligated with a silk suture (size 6-0). Tannic acid (40 mg) was dissolved in 1.0 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.01 M ascorbic acid, and the solution was purged with nitrogen to prevent oxidation before being introduced into the insect. Using a Hamilton microsyringe (10 μl) held with a micromanipulator, 2.0 μl of phosphate buffer (N = 3) or tannic acid solution (N = 10) was injected through the open (anterior) end of the midgut into the endoperitrophic space. A suture was used to clamp the anterior end of the midgut around the needle of the syringe to prevent leakage of the injected polyphenol solution. The anterior midgut was then ligated, and a small hole (0.9 ± 0.1 mm^2) was cut through the midgut wall to expose the peritrophic envelope. After rinsing the gut preparation for approximately 10 sec in each of three beakers of Tris buffer (described above), each gut preparation was placed in 1.5 ml of Tris buffer in the well of a tissue culture plate and incubated for 1.5-2 hr. Following incubation, the incubating solutions (1.5 ml) were transferred to test tubes, frozen (−20°C) and lyophilized. The contents of the midgut of each insect was extracted twice in 1.0 ml of 70% acetone (containing 0.001 M ascorbic acid) for 30 min at 22°C. Extracts were combined and centrifuged (4 min, 13,600g), and the supernatant solutions were concentrated under a stream of nitrogen. Concentrated extracts were lyophilized and stored at −20°C in a dessicator until they were analyzed with HPLC. Lyophilized samples were resolubilized in 0.5 ml (for incubating solutions) or 1.0 ml (for gut contents) of 20% acetonitrile containing 1% acetic acid, and filtered. Aliquots of samples (30 μl for incubating solutions, 20 μl for gut contents) and standards (25 μl) were analyzed with HPLC as described above. The mobile phase was 20% acetonitrile containing

1% acetic acid for incubating solutions, and 25% acetonitrile containing 1% acetic acid for gut contents.

This experiment was repeated using *P. nebrascensis*, with the following modifications: all insects were fed rye grass seedlings, distilled water (2 μl) was injected into the midguts of four insects (controls), tannic acid was injected into the midguts of six insects, gallic acid (2 μl, 40 mg/ml) was injected into the midguts of four insects, holes cut through the midgut wall averaged 2.2 ± 0.5 mm^2 and the mobile phase was 20% acetonitrile containing 1% acetic acid for all samples and standards.

Damage to the peritrophic envelopes during the dissection process was determined by observing whether a plume of dark gut fluid leaked from the exposed area. Any damage that was not detected during dissection was determined by examining the tannin permeation data for statistical outliers (Wilkinson, 1990). No outliers were observed in the data sets. Data from both experiments are presented as total μg polyphenols permeating the peritrophic envelope, since the amounts of polyphenols permeating the peritrophic envelope were not significantly correlated with either incubation time or hole diameter.

RESULTS

Composition of Tannins. Using either normal- or reverse-phase HPLC, the components of tannic acid elute in order of increasing number of galloyl esters (Figures 1 and 2; Hagerman et al., 1992). The conditions chosen for reverse-phase HPLC did not resolve the components of the tannic acid completely, but allowed for the higher molecular weight components to be measured (e.g., peak h; Figure 2). Isomers of several polygalloyl glucose esters were resolved as split peaks (Figure 2). Tannic acid from *Q. infectoria* (Sigma) contained an average of 10.2% trigalloyl glucose (peaks b and c), 21.1% tetragalloyl glucose (peaks d and e), 24.6% pentagalloyl glucose (peaks f and g), and 20.2% hexagalloyl glucose (peak h). The tannic acid used in this study also contained an average of 4.4% gallic acid (peak a). Mono- and digalloyl glucose and galloyl glucose esters larger than hexagalloyl glucose, which collectively account for the remaining 19.5% of the tannic acid, could not be quantified accurately with reverse-phase HPLC. Fireweed tannin contained oenothein B (>80%), gallic acid (<1%), and some lower molecular weight compounds that were not identified.

Tannin Budgets. Only a small fraction of the tannins ingested by either *M. sanguinipes* (5–25%) or *P. nebrascensis* (22%) could be recovered from their frass (Table 1). The tannins that were recovered from the frass of *P. nebrascensis* consisted primarily of tetragalloyl glucose, whereas low concentrations of several gallotannins were present in the frass of *M. sanguinipes*, but these were poorly resolved using HPLC.

Tannin Hydrolysis. In *M. sanguinipes* 12.1-46.9%, and in *P. nebrascensis* 82.0%, of the galloyl ester groups of the ingested tannins were hydrolyzed (Table 2). In *M. sanguinipes* tannic acid was more extensively hydrolyzed when ingested in an artificial diet than when ingested on lettuce leaves, and tannic acid was more susceptible to hydrolysis than fireweed tannin.

Changes in Tannic Acid and Gallic Acid Concentrations Along the Gut. The tannic acid content of the foregut was substantially less than that of the diet, identifying the foregut as a major site of transformation of tannic acid (Figure 3). In contrast, the gallic acid content of the foregut was 3.6 times higher than in the food, demonstrating that hydrolysis accounts for the loss of some tannic acid in the foregut. However, we calculate that hydrolysis can

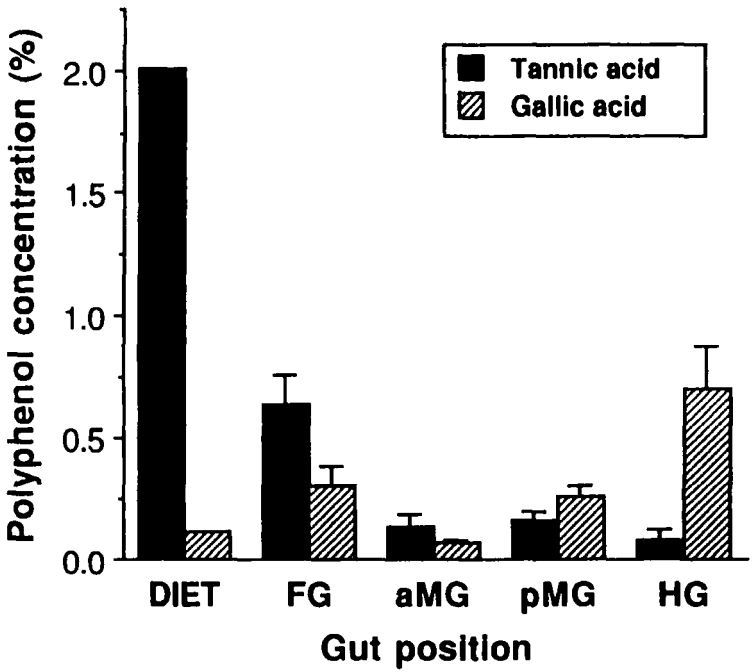

FIG. 3. Change in the percentage (DW) of tannic acid and gallic acid in artificial diet passing through the guts of *Melanoplus sanguinipes*. Gallic acid in the diet is an impurity in tannic acid. The percentage of polyphenols in the absorptive regions of the gut (midgut and hindgut) were transformed to the percentage of polyphenols in an equivalent mass of food, using the expression: (μg polyphenol)/[(μg sample/(1 − 0.559)], where 0.559 is the average approximate digestibility of the food. FG = foregut, aMG = anterior midgut, pMG = posterior midgut, and HG = hindgut.

TABLE 1. DRY MASS BUDGETS FOR TANNIC ACID (TA) AND FIREWEED TANNIN (FWT) INGESTED BY *Melanoplus sanguinipes* AND *Phoetaliotes nebrascensis*[a]

Diet (N)	Tannin ingested	Tannin egested	Percent tannin recovered
Melanoplus sanguinipes			
TA in artificial diet[b] (12)	666.3 ± 74.9	153.1 ± 23.6	22.5 ± 2.4
TA in artificial diet[c] (9)	34.4 ± 4.7	7.4 ± 0.7	24.7 ± 3.6
FWT in artificial diet (11)	1402.7 ± 91.2	166.4 ± 21.7	12.3 ± 1.6
TA on lettuce leaves[d] (12)	398.5 ± 25.1	21.6 ± 4.2	5.3 ± 1.0
TA on lettuce leaves[d] (10)	478.9 ± 49.4	22.7 ± 7.4	5.4 ± 1.6
Phoetaliotes nebrascensis			
TA on rye leaves[d] (9)	420.8 ± 8.7	94.3 ± 21.4	22.5 ± 5.2

[a] Data expressed as mean μg/insect ± SE.
[b] TA components eluting between 4–8 min (Figure 2) from a Vydac C-18 column were quantified.
[c] TA ingested was estimated from the weight of frass produced and an average approximate digestibility of 55.9%. TA egested was measured in frass pellets dissected from the hindgut. TA components eluting between 4.5–10 min (Figure 2) from a Vydac C-18 column were quantified.
[d] TA components eluting between 5–13 min (Figure 2) from a Vydac C-18 column were quantified.

TABLE 2. HYDROLYSIS OF TANNIC ACID (TA) AND FIREWEED TANNIN (FWT) DURING PASSAGE THROUGH THE GUTS OF *Melanoplus sanguinipes* AND *Phoetaliotes nebrascensis*[a]

Diet (N)	Total tannin ingested	Net GA egested[b]	Percent galloyl esters hydrolyzed[c]
Melanoplus sanguinipes			
TA in artificial diet (9)	40.1 ± 5.4[d]	8.3 ± 2.0	46.9 ± 12.3
FWT in artificial diet (11)	1402.7 ± 91.2[e]	52.7 ± 5.3	19.7 ± 1.6
TA on lettuce leaves (12)	526.8 ± 33.2[e]	34.6 ± 19.3	12.1 ± 6.6
TA on lettuce leaves (10)	694.3 ± 66.5[e]	32.1 ± 17.8	13.0 ± 7.6
Phoetaliotes nebrascensis			
TA on rye leaves (9)	438.3 ± 8.7[f]	250.3 ± 28.7	82.0 ± 8.2

[a] Data expressed as mean μg/insect ± SE.
[b] Calculated from the expression (μg GA egested) − [(μg GA ingested) × (%recovery of ingested GA)].
[c] Calculated from the expression (net μg GA produced)/[(μg tannin ingested) × (μg GA produced per μg tannin hydrolyzed) × (%recovery of ingested GA)]. TA yields 69% GA and FWT yields 22% GA upon hydrolysis. The percent recovery of GA ingested in artificial diet by *M. sanguinipes* was 81.5, and 79.8 when fed on lettuce. In *P. nebrascensis* all GA was recovered.
[d] TA ingested was calculated from the weight of frass produced and an average approximate digestibility of 55.9%.
[e] Tannin ingested was calculated from the %DW of tannin added to the food and the amount of food consumed.

account for no more than 45% (±7.6 SE) of the tannic acid lost from the diet in the foregut. There was a further drop in the concentration of tannic acid in the anterior midgut, identifying this region as an additional site of transformation of tannic acid. The correct concentration of tannic acid and gallic acid in the anterior midgut depends upon the extent of nutrient assimilation in this region, and may be less if an assimilation efficiency of 55.9%, which we used to correct the food mass, is excessive. By contrast, if no nutrient assimilation had occurred in the anterior midgut, then the percent tannic acid and gallic acid in the anterior midgut would have been 0.88 ± 0.15% and 0.33 ± 0.04%, respectively. The large increase in the concentration of gallic acid in the hindgut was presumably due to the excretion of assimilated gallic acid by the Malpighian tubules.

Gut pH and Redox Conditions. The pH throughout the guts of *M. sanguinipes* and *P. nebrascensis* is mildly acidic to neutral (Table 3). The redox conditions measured throughout the guts of both species are oxidizing (positive Eh, pH + pe > 9) (Table 3).

Polyphenol Oxidation. Polyphenol oxidation generates brown pigments (Hathway and Seakins, 1957; Thomson, 1962; Leatham et al., 1980; Igarashi and Yasui, 1985; Cilliers and Singleton, 1989). There was a significant increase ($P < 0.01$) in the amounts of brown pigments in the frass of insects that consumed tannic acid or fireweed tannin in an artificial diet (Table 4). There was no significant increase in brown pigment formation in *M. sanguinipes* that ingested gallic acid in artificial diet, consistent with the high recovery of ingested gallic acid in the frass (81.6%). Because of the high background absorbance in plant extracts, this method could not be used as an indicator of polyphenol oxidation in insects fed treated foliage.

Adsorption of Tannic Acid on the Peritrophic Envelope. When *M. sanguinipes* was fed tannic acid-coated lettuce, less than 1% of the total tannic acid

TABLE 3. pH AND REDOX CONDITIONS IN THE GUTS OF *Melanoplus sanguinipes* AND *Phoetaliotes nebrascensis*[a]

Gut position (N)	pH	Eh (mV)	pe	pH + pe
M. sanguinipes				
Foregut (14)	5.52 ± 0.07	270 ± 20	4.55 ± 0.33	10.07 ± 0.33
Midgut (14)	6.75 ± 0.14	168 ± 18	2.84 ± 0.31	9.58 ± 0.30
Hindgut (8)	6.80 ± 0.08	238 ± 16	4.01 ± 0.28	10.81 ± 0.28
P. nebrascensis				
Foregut (7)	6.03 ± 0.08	187 ± 7	3.17 ± 0.12	9.19 ± 0.12
Midgut (7)	7.12 ± 0.17	118 ± 8	1.99 ± 0.14	9.11 ± 0.22
Hindgut (7)	6.11 ± 0.14	213 ± 9	3.60 ± 0.16	9.71 ± 0.09

[a] Data are presented as mean ± SE.

TABLE 4. BROWN PIGMENTS (A_{420}) PRODUCED IN *Melanoplus sanguinipes* FED TANNIC ACID, FIREWEED TANNIN, AND GALLIC ACID IN ARTIFICIAL DIET[a]

Polyphenol	Control (N) (A_{420}/mg)	Treatment (N) (A_{420}/mg)
Tannic acid (2.6%)	0.008 ± 0.001 (6)	0.028 ± 0.009 (12)**
Fireweed tannin (2.6%)	0.029 ± 0.003 (10)	0.048 ± 0.004 (11)**
Gallic acid (2.2%)	0.016 ± 0.003 (2)	0.013 ± 0.002 (7)

[a]Data are presented as mean ± SE. **indicates $P < 0.01$ (Mann-Whitney U-test).

present in its midgut was adsorbed on the peritrophic envelope (Table 5). The tannic acid that was adsorbed on the peritrophic envelope of *M. sanguinipes* consisted of roughly equal amounts of tetragalloyl and hexagalloyl glucose.

Permeability of the Peritrophic Envelope to Polyphenols. Several gallotannins were found to permeate the peritrophic envelope of *M. sanguinipes* (Table 6). For example, the peritrophic envelope was permeated by 10.8% of the tetragalloyl glucose present in the tannic acid that was injected into the midgut, or 15.0% of that which was still detectable in the midgut and incubating solution at the end of the incubation period. In *P. nebrascensis*, the peritrophic envelope was permeated by 19.8% of the tetragalloyl glucose that was initially injected into the midgut, or 26.9% of that which was still detectable in the midgut and incubating solution at the end of the incubation period. The peritrophic envelopes of both species were freely permeable to gallic acid (Table 6). When pure gallic acid (80 μg) was introduced into the midgut of *P. nebrascensis*, an average of 28.7 μg (35.9 ± 5.0%) diffused through the peritrophic envelope.

DISCUSSION

Most of the tannin ingested by *M. sanguinipes* (75–95%) and *P. nebrascensis* (78%) is removed during passage through the gut. In *M. sanguinipes*,

TABLE 5. ADSORPTION OF TANNIC ACID (TA) ON THE PERITROPHIC ENVELOPE (PE) OF *Melanoplus sanguinipes in vivo*[a]

TA in Midgut	PE Dry weight[b]	TA Adsorbed on PE
107.3 ± 10.3	97.8 ± 16.8	0.95 ± 0.24

[a]Data expressed as mean μg ± SE. N = 10.
[b]Does not include peritrophic envelope in the caeca.

TABLE 6. POLYPHENOLS IN TANNIC ACID RETAINED WITHIN OR PERMEATING THE PERITROPHIC ENVELOPES OF *Melanoplus sanguinipes* AND *Phoetaliotes nebrascensis*[a]

Polyphenol	μg Introduced into midgut	μg Retained within PE	μg Recovered in incubating solution
M. sanguinipes (10)			
Gallic acid	3.5	10.1 ± 1.9	6.6 ± 1.2
Tannin components	52.8	18.3 ± 1.3	2.3 ± 0.5
Tetragalloyl glucose	16.9	11.5 ± 1.2	1.8 ± 0.4
Pentagalloyl glucose	19.7	3.4 ± 0.6	0.01 ± 0.006
Hexagalloyl glucose	16.2	3.4 ± 0.8	0.4 ± 0.2
P. nebrascensis (6)			
Gallic acid	3.5	1.3 ± 0.2	2.9 ± 0.8
Tannin components	52.8	11.4 ± 2.4	4.0 ± 1.3
Tetragalloyl glucose	16.9	7.3 ± 0.6	3.3 ± 0.9
Pentagalloyl glucose	19.7	2.1 ± 0.8	0.6 ± 0.4
Hexagalloyl glucose	16.2	2.0 ± 1.1	0

[a]Tannic acid was introduced into the midguts of insects by injecting 2 μl of a 4% solution of tannic acid in pH 7 phosphate buffer. Gut preparations were incubated for 1.5–2 hr. Data are presented as mean ± SE.

the primary sites of tannin removal are the foregut and the anterior midgut. We can envision *a priori* three processes that would result in the removal of tannins from an insect's gut: hydrolysis, adsorption on the peritrophic envelope, and oxidation. The first two are potential detoxification mechanisms. By contrast, the third is a process that potentially contributes to tannin toxicity.

In agreement with earlier studies (Bernays, 1978; Bernays and Chamberlain, 1980), we found that gallotannins are hydrolyzed in the guts of polyphagous grasshoppers. The extent of hydrolysis was much less in *M. sanguinipes* than in *P. nebrascensis*, and varied widely depending upon the type of tannin and nature of the diet in which it was ingested. The limited extent of tannin hydrolysis in *M. sanguinipes* argues strongly against the idea that this transformation plays a significant role in the detoxification of tannins in this species. For hydrolysis to result in detoxification, it must be extensive and it must result in the formation of nontoxic products. Partial hydrolysis, in which one gallotannin is converted into another with fewer galloyl groups, will be an effective detoxification mechanism only if the lower molecular weight gallotannin is significantly less toxic than the higher molecular weight analogue. The failure of partial hydrolysis to protect an insect from tannins is illustrated by the tannin-sensitivity of *Locusta migratoria*, a grasshopper in which 43% of the galloyl groups of ingested tannic acid are hydrolyzed (Bernays, 1978). We conclude, therefore, that hydrolysis is not a general mechanism that is sufficient to explain the widespread tolerance among polyphagous grasshoppers of hydrolyzable tannins.

Adsorption on the peritrophic envelope is a second mechanism by which tannins might be removed from the gut milieu, thereby protecting grasshoppers from the potential toxic effects of ingested tannins (Bernays and Chamberlain, 1980). We have not confirmed the earlier report that significant quantities of tannic acid are adsorbed by the peritrophic envelopes of polyphagous grasshoppers. Whereas Bernays and Chamberlain (1980) reported that the peritrophic envelope of *Schistocerca gregaria* adsorbed 100% of its weight in tannic acid *in vivo*, we found that the peritrophic envelope of *M. sanguinipes* adsorbed less than 1% of its weight in tannic acid. The different results obtained in the two studies may reflect the selection of different species or the use of different analytical methods. In the earlier study, adsorbed tannic acid was estimated by measuring the A_{270} of the HCl-hydrolyzate of the peritrophic envelopes of insects fed tannic acid-treated foliage. However, it was not established whether the A_{270} was due to gallic acid or to other compounds solubilized during hydrolysis. The method used in the present study (HPLC) entailed both the identification and quantification of specific gallotannins adsorbed by the peritrophic envelope. Whether the different results are due to our use of different species or different techniques, the conclusion is the same: the removal of tannic acid from the gut lumen by adsorption on the peritrophic envelope is not a general mechanism explaining the widespread tolerance among polyphagous grasshoppers of hydrolyzable tannins.

In further contrast to the earlier report that the peritrophic envelopes of tannin-tolerant grasshoppers are impermeable to tannic acid (Bernays et al., 1980), we have found that some of the gallotannins in tannic acid readily move across the peritrophic envelopes of *M. sanguinipes* and *P. nebrascensis*. In previous studies, the impermeability of the peritrophic envelope to tannins was inferred from the absence of lesions in histological sections (Bernays et al., 1980; Steinly and Berenbaum, 1985). Alternatively, the permeability of the peritrophic envelope to tannins was inferred from the presence of midgut lesions, or from the accumulation of polyphenols in damaged midgut tissues. The validity of this indirect approach is questionable; it is possible that tannins could cross the peritrophic envelope but not damage, or accumulate in, midgut tissues. In this study, we evaluated the permeability of the peritrophic envelope to tannins by direct measurement of the amounts of specific gallotannins that diffused from the endo- to the ectoperitrophic space. From our observation that the peritrophic envelopes of *M. sanguinipes* and *P. nebrascensis* are permeable to some gallotannins, we conclude that the tannin-tolerance of generalist grasshoppers does not result simply from the containment of tannic acid within the endoperitrophic space.

In our studies of *Malacosoma disstria* and *Orgyia leucostigma* (Lepidoptera), we found that tannic acid was oxidized in the midgut of *M. disstria*, a tannin-sensitive species, but not in the midgut of *O. leucostigma*, a tannin-

tolerant species (Barbehenn and Martin, 1992, 1994). These findings lead us to expect that tannin-tolerance in polyphagous grasshoppers would be associated with a lack of oxidation of tannic acid in their guts. However, conditions in the guts of both species favor oxidation. Oxidizing redox conditions have also been found in the guts of *Locusta migratoria* (Bignell, 1984) and *Ageneotettix deorum* (Barbehenn, unpublished data), suggesting that oxidizing conditions are a common feature of the grasshopper gut. Further evidence that tannins are oxidized in the guts of *M. sanguinipes* is provided by the observation of elevated levels of brown pigments in the frass of insects feeding on tannin-containing diets. These measurements cannot be used to calculate the actual amount of polyphenol oxidized, since the relationship between the amount of absorption at 420 nm and the amount of polyphenol oxidized has not been determined. However, if oxidation accounts for the difference between the amount of tannin ingested and the amount that can be recovered from the frass plus the amount determined to have been hydrolyzed, then the percent oxidized was substantial in *M. sanguinipes*: 28% in artificial diet and 83% on foliage. Thus, the ability of some polyphagous grasshoppers to tolerate ingested tannins results from their protection from the consequences of polyphenol oxidation, not from the absence of oxidation.

Antioxidant systems are well known in insects (Ahmad, 1995; Felton, 1995; Felton and Summers, 1995). Gut tissues may be protected from reactive oxygen species by enzymes, such as catalase and glutathione transferase peroxidase, and antioxidants, such as ascorbic acid. The polysaccharides and proteoglycans of the peritrophic envelope may also trap oxygen radicals generated in the midgut lumen (Felton and Summers, 1995). Felton and Summers (1995) showed that oxidative damage to midgut tissues was reduced *in vitro* in proportion to the mass of peritrophic envelope present. Thus, the hypothesis that the peritrophic envelope plays a critical role in protecting polyphagous grasshoppers from tannins remains a viable one, although the role may be to scavenge oxygen radicals, rather than to contain tannins within the endoperitrophic space. This possibility seems especially plausible considering the finding of Bernays and Simpson (1989) that the peritrophic envelopes of tannin-tolerant grasshoppers are thicker, and potentially capable of scavenging more oxygen radicals, than those of tannin-sensitive species.

Finally, we propose that the structural organization of digestion in grasshoppers may contribute to the ability of some species to tolerate ingested tannins. The foregut is not only a region in which a substantial amount of digestion occurs (Robinson, 1964; Price and Robinson, 1966; Evans and Payne, 1964; Droste and Zebe, 1974), but it is also the most active region for the hydrolysis, and probably the oxidation, of ingested tannic acid in *M. sanguinipes*. Grasshopper foreguts contain copious quantities of dark gut fluid ("tobacco juice"). The dark color of this fluid is consistent with the occurrence of polyphenol

oxidation in the foregut. In *M. sanguinipes*, most of the oxidation of tannic acid appears to take place in the foregut, since hydrolysis can account for only 45% of the tannic acid lost before ingested food reaches the anterior midgut. Because the foregut is lined with impermeable cuticle (Maddrell and Gardiner, 1980; Chapman, 1982), polyphenol oxidation in this region would not pose a threat to sensitive gut tissues, as it would in the midgut. Thus, the grasshopper foregut may function as an oxidation-resistant reaction vessel, in which the oxidation of ingested tannins occurs prior to their passage into the more sensitive midgut.

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MEDIATION OF CARDIAC GLYCOSIDE INSENSITIVITY IN THE MONARCH BUTTERFLY (*Danaus plexippus*): ROLE OF AN AMINO ACID SUBSTITUTION IN THE OUABAIN BINDING SITE OF NA^+,K^+-ATPase

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Abstract—The Monarch butterfly (*Danaus plexippus*) sequesters cardiac glycosides (CG) for its chemical defense against predators. Larvae and adults of this butterfly are insensitive towards dietary cardiac glycosides, whereas other Lepidoptera are sensitive and intoxicated by ouabain. Ouabain inhibits Na^+,K^+-ATPase by binding to its α-subunit. We have amplified and cloned the DNA-sequence encoding the respective ouabain binding site. Instead of the amino acid asparagine at position 122 in ouabain-sensitive insects, the Monarch has a histidine in the putative ouabain binding site, which consists of 12 amino acids. Starting with the CG-sensitive Na^+,K^+-ATPase gene from *Drosophila*, we converted pos. 122 to a histidine residue as in *Danaus plexippus* by site-directed mutagenesis. Human embryonic kidney cells (HEK) (which are sensitive to ouabain) were transfected with the mutated Na^+,K^+-ATPase gene in a pSVDF-expression vector and showed a transient expression of the mutated *Drosophila* Na^+,K^+-ATPase. When treated with ouabain, the transfected cells tolerated ouabain at a concentration of 50 mM, whereas untransformed controls or controls transfected with the unmutated *Drosophila* gene, showed a substantial mortality. This result implies that the asparagine to histidine exchange contributes to ouabain insensitivity in the Monarch. In two other CG-sequestering insects, e.g., *Danaus gilippus* and *Syntomeida epilais*, the pattern of amino acid substitution differed, indicating that the Monarch has acquired this mutation independently during evolution.

Key Words—*Danaus plexippus*, *Danaus gilippus*, *Syntomeida epilais*, *Syntomis mogadorensis*, cardiac glycosides, Na^+,K^+-ATPase, target site modi-

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fication, insensitivity, amino acid substitution, sequestration, gene expression, site-directed mutation.

INTRODUCTION

Cardiac glycosides (CGs) are produced in many unrelated plant families, such as the Scrophulariaceae, Apocynaceae, Asclepiadaceae, Ranunculaceae, Brassicaceae, Liliaceae, Celastraceae, and few others. Even a few animals, such as toads and beetles, can produce their own CGs (Harborne, 1993; Teuscher and Lindequist, 1987).

The molecular target of CGs is the Na^+,K^+-ATPase. CGs inhibit this ion pump and thus destroy the ion gradients that are necessary for many cellular functions, including neuronal activity, secondary active transport systems and muscle contraction. These activities would explain the general toxicity of CGs in animals and their use as arrow poisons in many parts of Africa (Neuwinger, 1994). CGs are widely employed therapeutic agents; in the heart muscle the partial inhibition of Na^+,K^+-ATPase results in higher Na^+ concentration in muscle cells, which in turn activates a Na^+/Ca^{++} channel. Elevated Ca^{++} levels directly influence muscle contraction by activation of the tropomyosin/myosin/actin system.

A number of insects, especially moths and butterflies, sequester the defence compounds of their host plants and use them for their own protection against predators (reviews in Bernays and Chapman, 1994; Brown and Trigo, 1995; Harborne, 1993; Rosenthal and Berenbaum, 1991; 1992; Wink, 1993). The pioneering works of M. Rothschild, T. Reichstein, L. Brower, and coworkers, have established cardiac glycosides (CGs) as one of the best studied groups of secondary metabolites which are widely used as acquired defense compounds in insects (for review see: Brower, 1984; Brown and Trigo, 1995; Harborne, 1993; Rosenthal and Berenbaum, 1991; Rothschild, 1972b; Rothschild et al., 1972; Duffey and Scudder, 1972).

These specialized insects, which are often aposematically colored, are able to take up and store plant-derived CGs. Examples include grasshoppers (e.g., *Poekilocerus*, *Phytmaeteus*; Euw et al, 1967; Harborne, 1993; Rothschild, 1966; Rothschild, 1972b), aphids (e.g., *Aphis nerium*; Malcolm, 1990; Rothschild et al., 1970), lygaeid bugs (e.g., *Oncopeltus*, *Caenocoris*, *Spilostethus*, *Lygaeus*, *Apterola*, *Arocatus*, *Aspilocoryphus*, *Aulacopeltus*, *Graptostethus*, *Haemobaphus*, *Lygaeospilus*, *Melanerythrus*, *Microspilus*, *Horvathiolus*; Euw et al., 1971; Malcolm, 1990; Rothschild, 1972b; Rothschild et al., 1971; Scudder et al., 1986), beetles (*Tetraopes*, *Epicauta*; Rothschild, 1972b). Diptera (*Zenilla*; Rothschild, 1972b) and Lepidoptera (e.g., *Danaus*, *Syntomeida*, *Euchaetias*, *Arctia*, *Empyreuma*; Harborne, 1993; Nickisch-Rosenegk et al., 1990; Roth-

schild, 1972a,b; Rothschild et al., 1973). Insects sequestering CGs are usually protected against predators such as birds (Brower, 1984; Brower and Fink, 1985; Brower and Van Zandt Brower, 1964; Brower et al., 1968, 1975, 1982; Cohen, 1985; Rothschild, 1966; 1972a; Reichstein et al., 1968; Seiber et al., 1980, 1986; Ritland, 1991), although specialists can overcome the defense barrier (Fink and Brower, 1981; Glendinning, 1992).

Among organisms that sequester CGs, the Monarch is probably the best-studied taxon. Larvae of *Danaus plexippus* L. feed on milkweed plants of the genus *Asclepias*. Sequestration and storage of cardiac glycosides by monarchs is a selective process in both quantitative and qualitative terms (Brower et al., 1982; Lynch and Martin, 1987; Martin and Lynch, 1988; Malcolm, 1990; Malcolm and Brower, 1989; Reichstein et al., 1968; Roeske et al., 1976; Seiber et al.; 1980, 1986). Monarchs appear to regulate their overall content of cardiac glycosides in such a way that an appropriate cardenolide capacity necessary for chemical defense is achieved, although a substantial part of the monarch population has a low cardenolide content (Brower et al., 1972; Brower and Mofitt, 1974; Ritland, 1991). Out of the complex pattern of cardenolides that are present in food plants, the butterflies selectively store polar cardenolides but not the more lipophilic apolar glycosides (Brower, 1984; Brower et al., 1988; Groeneveld et al., 1990; Malcolm et al., 1989). Since many CGs are polar compounds, they cannot pass biomembranes by simple diffusion. A carrier-mediated uptake system which could explain the observed selectivity has been proposed for CG uptake in *D. plexippus* (Frick and Wink, 1995) and also in other CG-insects such as *Syntomeida epilais* (Nickisch-Rosenegk et al., 1990), and *Oncopeltus fasciatus* (Detzel and Wink, 1995). Cardiac glycosides are apparently not stored in all tissues of the monarch, but preferentially in the exoskeleton of the butterfly, i.e., the wings, the cuticle and in the fat body (Brower and Glazier, 1975; Brower et al., 1988; Frick and Wink, 1995; Seiber et al., 1986). This peripheral storage seems to provide a chemical defense barrier against non-adapted enemies, but some predators (birds and mice) can learn to avoid the cardenolide-rich tissues (Brower et al., 1988; Fink and Brower, 1981; Glendinning, 1990). For example, feeding experiments in three mouse species (*Peroscomys melanotis*, *P. aztecus*, and *Reithrodontomys sumichrasti*) with intact monarch butterflies of differing CG content showed that CG-rich cuticular material (wings, integuments) was clearly rejected, whereas CG-free butterflies and tissues of low CG content were preferred (Glendinning, 1990). Mice picked up CG-rich butterflies in their mouth, but immediately dropped them. No signs of biting and chewing were observed, but it appeared that they tasted the cuticle.

It was shown experimentally (C. Frick and M. Wink, unpublished), that *D. plexippus* larvae can tolerate CG doses exceeding 1200 mg/kg body weight (which would kill almost any other animal). Therefore, as a prerequisite for CG utilization, *Danaus* must have evolved either insensitivity in its Na^+,K^+-ATPase

and/or detoxification mechanisms (such as storage in the integument). Indeed, its Na^+,K^+-ATPase cannot be inhibited by CGs (Holzinger et al. 1992; Vaughan and Jungreis, 1977). How can we understand the insensitivity of Na^+,K^+-ATPase at the molecular level? The Na^+,K^+-ATPase consists of two subunits, a catalytic and ouabain-binding α-subunit with 1016 amino acids and a β-subunit with 302 amino acids (Lingrel et al., 1990; Shull et al., 1985). The α-subunit shows eight transmembrane domains and the main ouabain binding site is probably positioned in the extracellular loop between the transmembrane domain H1 and H2 (amino acid position 111–122; Figure 1a) (Lingrel et al., 1990). This putative ouabain binding site covers 36 nucleotides. In rats, a ouabain insensitive enzyme has been reported in which the substitution of arginine for glutamine at position 111 and aspartic acid for asparagine at position 122 is responsible for CG insensitivity, as compared to sensitive Na^+, K^+-ATPases from man or sheep (Lingrel et al., 1990). Recently, evidence was also presented that the first transmembrane segment of Na^+,K^+-ATPase confers ouabain resistance to MDCK canine cells (Canessa et al., 1992) and digoxin/digitoxin insensitivity (Askew and Lingrel, 1994).

A few years ago we started to analyze the apparent CG-insensitivity of CG-sequestering insects at the molecular level. Using the sequence information available (Lingrel et al., 1990; Shull et al., 1985), oligonucleotide primers were constructed to amplify the H1–H2-loop of Na^+,K^+-ATPase from sensitive and nonsensitive insects by PCR (Table 1). Nucleotide sequences obtained from the lepidopterans *Danaus plexippus*, *Manduca sexta*, and *Creatonotos transiens* showed a high homology to those of the α-subunits from other organisms, especially to that from *Drosophila* (Tables 1, 2). Introns of different lengths were found between the amino acid positions 109 and 110, just adjacent to the ouabain binding site (Holzinger et al., 1992).

Comparing the nucleotide sequences and the derived amino acid sequences of the ouabain binding site (Table 2), 9 of the 12 amino acids of the insects studied were identical. Whereas, substitutions at positions 115 and 119 are probably not significant, position 122 appears to be critical (Lingrel et al., 1990): A histidine is present in *D. plexippus* (Figure 1b) whereas ouabain-sensitive insects and vertebrates carry an asparagine instead. Since histidine changes the receptor properties to a high degree, we assume that this target site modification in Monarchs may lead to a ouabain insensitive Na^+,K^+-ATPase.

In this communication, we provide direct evidence that the amino acid substitution in the putative ouabain binding site (Holzinger et al., 1992), is indeed a critical mutation contributing to cardiac glycoside insensitivity in the Monarch. In addition, we have analyzed whether other insects which store cardiac glycosides, such as *Danaus gilippus* (Ritland, 1991) or *Syntomeida epilais* (Nickisch-Rosenegk et al., 1990; Rothschild et al., 1973), exhibit a similar adaptation.

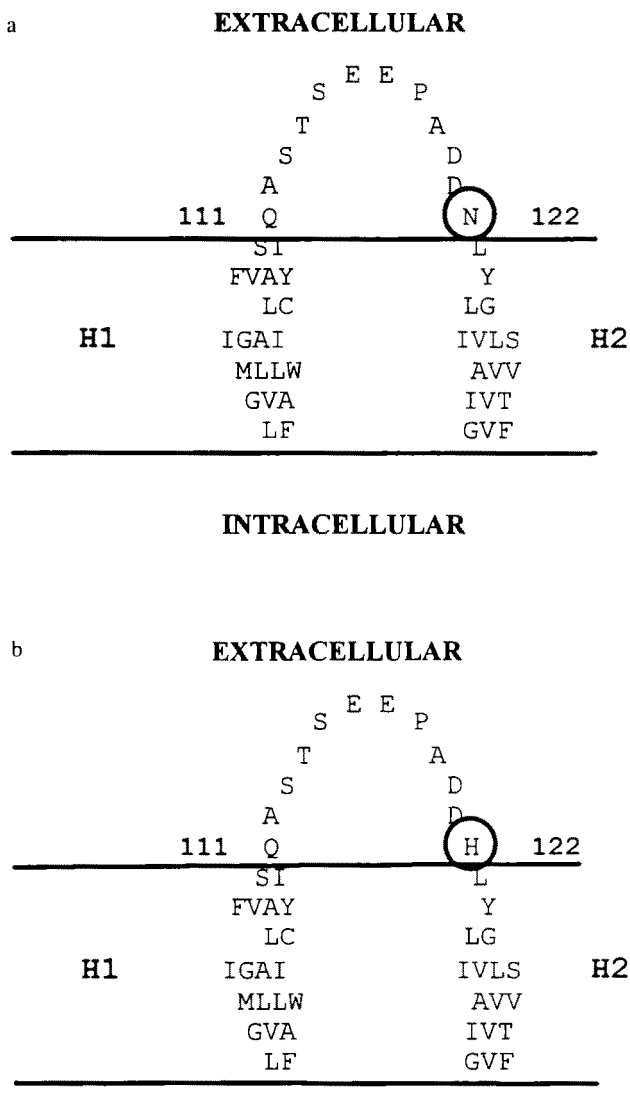

FIG. 1. H1-H2 region of *Drosophila* Na^+,K^+-ATPase α subunit. a. The circled amino acid was target of a point mutation; b. Asparagine was exchanged to histidine (circle) by site-directed mutagenesis, resulting in an extracellular loop similar to that of the Monarch.

TABLE 1. PARTIAL NUCLEOTIDE SEQUENCE OF THE H1 AND H2 DOMAIN OF THE α-SUBUNIT OF Na^+, K^+-ATPASE OF *Danaus plexippus*, *D. gilippus**, *Manduca sexta*, *Creatonotos transiens*, *Syntomeida epilais**, *Syntomis mogadorensis**, AND *Drosophila*

	Primer A					
	1		21		41	
Drosophila	CTGTGGATCG	GTGCTATTCT	CTGCTTTGTG	GCCTATTCTA	TCCAGGCCAG	CACCAGCGAG
D. plexippus	CTGTGGATTG	GAGAGATTCT	ATGCTTTATT	GCCTATGGTA	TTCAGGCGAG	TACTGTTGAA
D. gilippus	CTGTGGATTG	GAGAGATTCT	ATGCTTTATT	GCCTATGGGA	TTCAGGCGAG	TACTGTTGAA
M. sexta	CTGTGGATCG	GTGCGATTCT	TTGCTTTATT	GCATATGGAA	TCCAGGCGAG	TACCGTAGAA
C. transiens	CTGTGGATTG	GAGAGATTCT	ATGCTTTATT	GCCTATGGAA	TTCAGGCTAG	TACCGTAGAG
S. epilais	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNCAGGCGAG	TACCGAAGAA
S. mogadorensis	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNCTACGGAA	TTCAGGCGAG	CACCGTAGAA
	61		81		101	
Drosophila	GAGCCGGCCG	ACGATAATTT	GTATCTGGGT	ATTGTACTTT	CCGCTGTCGT	CATCGTGACG
D. plexippus	GAACCCTCGG	ACGACCACTT	GTATCTCGGA	ATCGTATTGG	CGGCTGTCGT	TATCGTGACT
D. gilippus	GAACCCTCGG	ATGACAACTT	GTATCTCGGA	ATCGTATTGG	CTTCTGTCGT	TATCGTGACT
M. sexta	GAACCCTCCG	ATGATAACTT	GTACCTTGGC	ATTGTATTGG	CGGCTGTCGT	GATCGTTACG
C. transiens	GAACCAGCGG	ATGACAATCT	TTACCTCGGC	ATCGTATTGG	CAGCTGTCGT	GATCGTTACG
S. epilais	GAACCTTCAG	ATGATAACTT	GTACCTNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN
S. mogadorensis	GAACCAGCGG	ATGACAACTT	GTACCTCGGC	ATTCTTTTGG	CAGCTGTTGT	AATCGTGACG
	121		141		161	
Drosophila	GGCGTTTTCT	CATACTATCA	GGAATCGAAG	AATTCTAAGA	TCATGGAATC	GTTCAAGAAC
D. plexippus	GGAATATTCT	CTTACTACCA	AGAAAGGAAG	TCTTCCAAGA	TCATGGAATC	CTTCAAAAAC
D. gilippus	GGAATCTTCT	CTTACTACNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN
M. sexta	GGTATATTCT	CATACTACCA	CGAAAGGAAG	TCATCTAAGA	TCATGGAATC	GTTCAAAAAC
C. transiens	GGTATCTTCT	CATACTATCA	GGAAAGGAAG	TCATCGAAGA	TCATGGAATC	GTTCAAAAAC
S. epilais	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN
S. mogadorensis	GGTATCTTCT	CATACTACCA	GGAAAGCAAG	TCATCGAAGA	TCATGGAATC	CTTCAAAAAC
					Primer B**	

D. plexippus						
INTRON	CTGGTAAGTT	GTAGGCTCTT	AATAACTTAA	GTCATCTATT	ATTTATTTTT	GTGAGATGTT
	GAATATATTA	CAACAACATT	ATGAAACTC			
D. gilippus						
INTRON	GATTGTGGTA	GTGTAGCTTT	AATACTTACG	GTCATCTATA	ATAAATTTTT	GAATATATTA
	TAATAATCTC	ATAAAACTC				
M. sexta						
INTRON	CAGGTAAGTT	AATAAGCATG	TCAATTACAT	ATAGCGTTAT	CAACGAAAAA	ATATAATTTC
	GGAATAAAAT	AATCCTCACA	TAAGACAGCC	TACCACGATG	GACTCCTTTG	GGTGAATATT
	CCCATATCAA	ATAAAATTCT	TTTACTTAAT	TTATACCATC	ATCACTTAAC	AAACGAAAA
	CATCAATGAA	TTGGCTGGCT	GGTCTGATCA	TCAGTATAAA	CTGAAGTTTT	TGTCTTC
C. transiens						
INTRON	CAGGTAAGTA	ACGCAACAAA	TACAATATAC	TCAATAGCAA	GTATACGACT	TATAATTAAT
	ATGGCAAATA	CTCCTC				
S. epilais						
INTRON	TTTATTTACT	CATTTAGTGA	CAGAATATTA	AATGTAGACA	CGTTCACCCT	C
S. mogadorensis						
INTRON	CAGGTCAGTG	AGAGCACCTT	TTTTCAAAGC	AAATGTCTTT	GAGTATAGAG	CTTATACCAA
		AAATATTCGA	CACTTC			

* = new, **the original primer was 5 bases longer at the 5′ end as indicated here.
N = not recorded; the ouabain binding site (pos. 43-79) is printed in bold; introns were inserted between nucleotide positions 42 and 43.

TABLE 2. DERIVED AMINO ACID SEQUENCES OF THE OUABAIN BINDING SITE OF OUABAIN-SENSITIVE AND INSENSITIVE ORGANISMS

Sensitive enzymes:												
	111				115					120		122
Human	Gln	Ala	Ala	Thr	Glu	Glu	Glu	Pro	Gln	Asn	Asp	Asn
Sheep	Gln	Ala	Ala	Thr	Glu	Glu	Glu	Pro	Gln	Asn	Asp	Asn
Drosophila	Gln	Ala	Ser	Thr	Ser	Glu	Glu	Pro	Ala	Asp	Asp	Asn
M. sexta	Gln	Ala	Ser	Thr	Val	Glu	Glu	Pro	Ser	Asp	Asp	Asn
C. transiens	Gln	Ala	Ser	Thr	Val	Glu	Glu	Pro	Ala	Asp	Asp	Asn
S. mogadorensis	Gln	Ala	Ser	Thr	Val	Glu	Glu	Pro	Ala	Asp	Asp	Asn
Insensitive enzymes:												
	111				115					120		122
Rat	Arg	Ser	Ala	Thr	Glu	Glu	Glu	Pro	Pro	Asn	Asp	Asp
D. plexippus	Gln	Ala	Ser	Thr	Val	Glu	Glu	Pro	Ser	Asp	Asp	**His**
D. gilippus	Gln	Ala	Ser	Thr	Val	Glu	Glu	Pro	Ser	Asp	Asp	Asn
S. epilais	Gln	Ala	Ser	Thr	Glu	Glu	Glu	Pro	Ser	Asp	Asp	Asn

MATERIAL AND METHODS

Animals

Eggs of *D. plexippus* came from a laboratory colony kept at the Boyce Thompson Institute for Plant Research at Ithaca (USA). In Germany larvae were reared on *Asclepias syriaca* and a few generations of Monarchs were grown in a greenhouse in the summers of 1991 to 1993. *D. gilippus* were obtained as adults; *S. mogadorensis* was reared on *Taraxacum* and *S. epilais* on *Nerium oleander*. The latter three species were provided by D. Schneider.

Molecular Characterisation of the Putative Ouabain Binding Site

DNA was isolated from adult animals of *D. plexippus*, *D. gilippus*, *Syntomis mogadorensis*, and *Syntomeida epilais* using the CTAB-method (Doyle and Doyle, 1990). About 1.5 μg of DNA was amplified by Taq-polymerase and the oligonucleotide primers A: 5′ CTG TGG ATC(T) GGT(A) GCT(GC) ATT CT 3′ and B: 5′ACC ATG TTC(T) TTG AAC(G) GAT TCC ATG ATC TT 3′. The PCR reaction was carried out in 50 μl reaction volume. This contained 1 μg genomic DNA, one unit of Taq DNA polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.01% gelatin (w/v), 0.1% Triton X-100, 50 μM dNTPs, and 10 pmoles of each primer. The solution was overlayed with 50 μl mineral oil. PCR temperature program was 4 min at 94°C, 30 cycles (1 min 95°C, 1 min 55°C, 1 min 72°C), finally, 5 min at 72°C. Non-incorporated nucleotides and primers were digested by phosphatase and exonuclease and PCR products were sequenced using the USB direct sequencing kit (USB, Braunschweig). Autoradiograms were evaluated manually.

Site-Directed Mutagenesis of the Na^+,K^+-ATPase Gene

For the conversion of ouabain binding site of the *Drosophila* gene to the corresponding one of *D. plexippus*, only a single base substitution in the codon encoding amino acid 122 is necessary. For creating this point mutation we used the PCR-based overlap extension strategy (Higuchi et al., 1988). The plasmid pSVDF (Takeyasu et al., 1988) containing the *Drosophila* Na^+,K^+-ATPase α subunit gene (obtained from D. M. Fambrough) was used as PCR template. Plasmid DNA was prepared by column chromatography (Qiagen). Both steps of the PCR reaction were carried out in 50 μl reaction volumes as described above. During the first step, 50 ng template DNA and the following primer pairs were used (modification underlined): Primer C 5′ CTCGCCCTCGCGGATGACGGT 3′ and D 5′ GCCGGCCGACGA-TCATTTGTATCTGGG 3′ in one tube, primer E 5′ GTTTTCCCAGTCAC-GACGTTGTA and F 5′ ACAAATGATCGTCGGCCGGCTCC 3′ in another

tube produced fragments of 156 and 562 bp length. After purification by agarose gel electrophoresis these products were used in equimolar amounts in the second step, by adding ten pmoles of primers C and E, which hybridize to the flanking sequences of the annealed products. The resulting cassette was purified, cut with Kpn I and ligated in pUC21 (Boehringer). Most of the derived clones contained the desired modification without further changes. The modified Kpn I fragment containing the sequence for the ouabain binding site was cut out of pUC21, separated by agarose gel electrophoresis, and ligated into the expression vector pSVDF construct.

Maintenance and Transfection of HEK-Cells

Human embryonic kidney cells (Eaton et al., 1986) were obtained from P. Seeburg (ZMBH, Heidelberg), and maintained in MEM with Earle's Salts (GIBCO), 10% FCS (Flow Laboratories), 1% (200 mM) L-glutamine, (Boehringer) and 1% penicilline-streptomycine (Flow Laboratories). Cells of half-confluent plates (10 cm, Costar) were transfected by using a modified $CaPO_4$ precipitation technique (Chen and Okayama, 1987). Fourty-eight hours after transfection, cells were washed with phosphate-buffered saline and scraped into a denaturing solution and further processed for RNA isolation. Expression of Na^+,K^+-ATPase in HEK cells was compared by dotblot analysis of RNA, following the procedure described by the manufacturer (Pall Biodyne B membrane).

RESULTS AND DISCUSSION

Transfection of HEK Cells with a Mutated Drosophila Na^+,K^+-ATPase Gene

As shown in Table 2, the amino acid sequence of the putative ouabain binding site of the *Drosophila* Na^+,K^+-ATPase is very similar to those of other CG-sensitive organisms. Using a site-directed mutation approach via specific PCR primer we have substituted the asparagine residue in position 122 with a histidine as in *D. plexippus* (Figures 1B and 2). The successful and correct mutation was corroborated by direct sequencing.

The gene was then ligated with the expression vector pSVDF (confirming the correct reading frame by sequence analysis), which is suitable for the transient expression of genes in animal cells (line HEK 293). As can be seen from a Northern hybridization (Figure 3) of RNA extracted from transfected and untransfected cells, which were hybridized with the *Drosophila* Na^+,K^+-ATPase gene, a transient expression of the construct takes place 24–72 hr (with a maximum after 48 hr) after transfection. The transfected cells were left for 24 hr before starting the ouabain experiments.

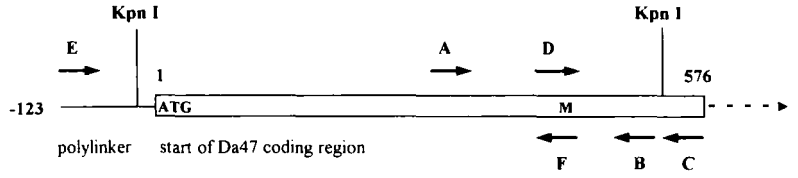

FIG. 2. Location and direction of the primers. The construct encloses a part of the polylinker and the start region (576 bp) of *Drosophila* Na^+,K^+-ATPase of the Da47 clone. Kpn I sites are at position −39 and 544 relative to the start codon. Primers A and B were used to amplify the ouabain binding site; primer pairs C/D and E/F were employed for site directed mutagenesis; the point mutation (M) is located at position 433. To obtain the "mut construct" the mutated fragment is cleaved at the Kpn I sites (size 583 bp) and ligated to the rest of the unmutated *Drosophila* gene which was also cleaved by Kpn I.

Human embryonic kidney cells (HEK cells) are sensitive towards ouabain. When it is added to the growth medium at 50 nM, cells do not grow but die. As can be seen from Figure 4, cell numbers (initial number was set at 100%) decreased by more than 54% 40 hr after adding 50 nM ouabain. However, when HEK cells were transfected with the Mut-construct (expressing the mutated Na^+,K^+-ATPase gene), cells did not die and the cell numbers were 106% 40 hr after ouabain was added (Figure 4). In a parallel experiment, cells were transfected with the unmutated *Drosophila* clone (Da47 cells); whereas cell

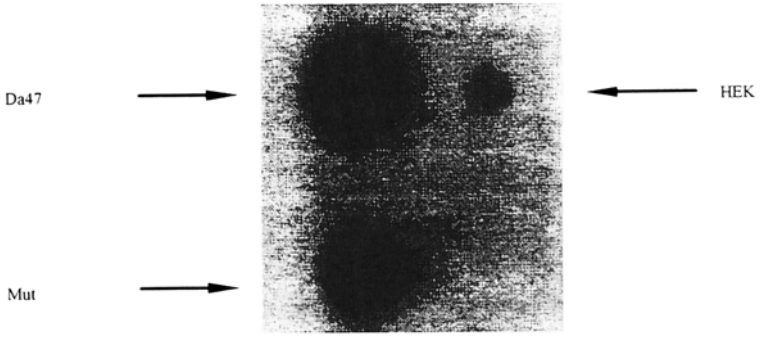

FIG. 3. Dotblot analysis of RNA from transfected and untransfected cells. Total RNA was prepared from HEK cells transfected with pSVDF plasmid DNA containing the wild type (Da47) or modified (Mut) Na^+,K^+-ATPase gene (α cDNA). After spotting 5 μg RNA onto the membrane, the blot was probed with an α subunit-specific PCR product. RNA from untransfected cells (HEK) show the normal expression level of the cell line.

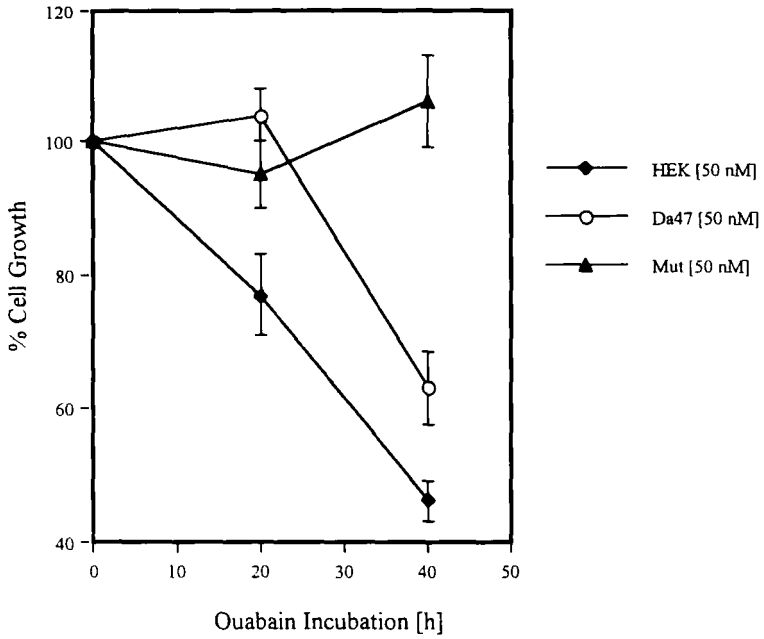

FIG. 4. Effect of ouabain on the growth of HEK cells. HEK cells transfected with pSVDF plasmid DNA containing the wild type (Da47) or modified (Mut) Na^+,K^+-ATPase gene (α cDNA) were grown in the presence of 50 nM ouabain. The cell number was determined at ouabain addition, 20 hr and 40 hr later and compared with untransfected control cells (HEK). Four plates were assayed for each set of experiments. The error bars show standard errors.

numbers were identical after 20 hr as compared to the Mut-clone, cell numbers dropped to ca 63% after 40 hr; a value that significantly differs from the Mut-value after 40 hr (Figure 4). In another set of experiments, ouabain was added at a concentration of 25 nM. Under these conditions cell numbers after 40 hr (initial number was set to 100%) were 262% in Mut-cells, 232% in Da47-cells and 182% in nontransformed HEK cells. These data clearly show that a point mutation at position 122 (substitution of his for asn) is indeed critical for ouabain toxicity, suggesting that this modification mediates cardiac glycosides insensitivity in the Monarch. However, it cannot be ruled out that an additional substitution (Schultheis et al., 1993) might also have occurred which could further amplify the insensitivity. A similar rationale was put forward by other authors who had transfected Hela cells with Na^+,K^+-ATPase genes in which the CG binding site had been modified by single amino acid substitutions (Lingrel et al., 1990; Askew and Lingrel, 1994; Jaisser et al., 1992; Schultheis et al., 1993).

Amino Acid Sequence of the Ouabain Binding Site in Danaus gilippus and Syntomeida epilais

Danaus gilippus and *Syntomeida epilais* are aposematically colored insects that store dietary CGs (Nickisch-Rosenegk et al., 1990; Ritland, 1991; Rothschild et al., 1973), whereas *Syntomis mogadorensis* (a related ctenuchid moth) eliminates CGs with the feces (Wink and Schneider, 1990). Using PCR primers developed to amplify the ouabain binding site of insects (Holzinger et al., 1992), we could obtain single PCR products which were directly sequenced. As can be seen from Table 1, these species also have an intron between positions 109 and 110, just flanking the ouabain binding site as was the case in *Manduca sexta*, *Creatonotos transiens* (Holzinger et al., 1992) and a few other insects (Emery et al., 1995). The derived amino acid sequence of the binding site of *D. gilippus* and of *S. mogadorensis* (Table 2), shows the typical pattern of CG-sensitive insects; i.e., the mutation found in the Monarch at position 122 could not be detected. In *S. epilais*, an amino acid substitution was visible at positions 115 and 119; since these substitutions also occur in sensitive enzymes of humans, sheep and other insects (Table 2) they cannot be responsible for CG-insensitivity. Because *D. gilippus* and *S. epilais* store CGs in the body and should be CG-insensitive, they must have developed a different target site modification.

CONCLUSIONS

Our experiments provide direct evidence that a single amino acid substitution in the ouabain binding site of Na^+,K^+-ATPase can confer resistance to ouabain in the Monarch. Genetically, such a mutation is straight-forward and only involves a point mutation of the corresponding gene. This mutation is an important requisite for CG sequestration and the utilization of CGs as acquired defense compounds; other adaptations apparently involve specific uptake and storage mechanisms.

Since many adaptations seem to be necessary for CG exploitation, it would be plausible to assume that these traits have been selected once during evolution and then maintained during further speciation processes. However, it is surprising that CG formation and sequestration can be found in many genetically unrelated organisms. CG insensitivity in the Monarch and in the other insects obviously depends (according to our molecular data) on different mechanisms; it is thus clearly a convergent trait and not derived through common ancestry. For CG production in genetically unrelated plant families and for CG sequestration in various groups of insects, we recently arrived at the same conclusion (Wink and v. Nickisch-Rosenegk, unpubl. data). CGs strongly interfere with an important molecular target of animals, i.e., the Na^+,K^+-ATPase and is thus a very potent toxin. Utilization of CGs for defence obviously grants a substantial

advantage for organisms, which could explain why CG adaptations became selected several times and independently during evolution.

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ISOLATION AND IDENTIFICATION OF (3-METHOXYPHENYL)ACETONITRILE AS A PHYTOTOXIN FROM MEADOWFOAM (*Limnanthes alba*) SEEDMEAL[1]

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Abstract—Ethyl ether, ethanol, and water extracts of meadowfoam (*Limnanthes alba* Hartweg ex. Benth.) seedmeal were prepared and bioassayed against velvetleaf (*Abutilon theophrasti* Medicus) and wheat (*Triticum aestivum* L. "Cardinal"). Both the ethyl ether and ethanol fractions, but not the water extract, inhibited velvetleaf and wheat radicle elongation. Fractionation of the extracts indicated that (3-methoxyphenyl)acetonitrile (3-MPAN) was the active compound from both extracts, comprising >97% of the active ethanol fraction. 3-Methoxybenzyl isothiocyanate, which had been previously shown to be the major breakdown product of glucolimnanthin, the major *L. alba* glucosinolate, was not detected in either extract. Radicle elongation of velvetleaf and wheat were inhibited by 3-MPAN with I_{50} (the concentration required to inhibit growth by 50%) values of approximately 4×10^{-4} M (velvetleaf) and 7×10^{-4} M (wheat).

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Key Words—Meadowfoam, *Limnanthes alba*, limnanthaceae, seedmeal, glucosinolate, glucolimnanthin, 3-methoxybenzyl isothiocyanate, (3-methoxyphenyl)acetonitrile, phytotoxicity, soil amendment.

INTRODUCTION

Meadowfoam (*Limnanthes alba* Hartweg ex. Benth.) is a commercial oilseed crop grown in the Willamette Valley of Oregon for its unusual fatty acids which are of value in the manufacture of cosmetics, lubricants, and plastics (Kleiman and Princen, 1991). At the present no uses have been found for the seedmeal remaining after oil extraction, so it has been discarded as waste. As part of an ongoing project examining alternative weed control methods, we are conducting research on the use of extracted meadowfoam seedmeal as a soil amendment/fertilizer. We observed in preliminary field studies that weed populations were reduced in fields to which seedmeal had been applied. Meadowfoam seeds contain the glucosinolate glucolimnanthin (Miller et al., 1964; Bartelt and Mikolajczak, 1989). Glucolimnanthin would not be expected to be extracted with the oil, as it is not soluble in the solvent (hexane) used for oil extraction and therefore, should be retained in the seedmeal. Glucosinolate degradation products include isothiocyanates, nitriles, and thiocyanates (Figure 1), although formation of thiocyanates is rare. Whether isothiocyanates or nitriles are formed depends on several factors, including prior treatment of the plant tissue, pH or type of glucosinolate present (VanEtten and Tookey, 1983). Some of these degradation products, especially certain isothiocyanates, have been shown to be potent biocides (Lewis and Papavizas, 1971; Brown et al., 1991; Gamliel and Stapleton, 1993; Brown and Morra, 1995; Delaquis and Mazza, 1995). Glucolimnanthin from unextracted *Limnanthes alba* seed has been shown to break down to form primarily 3-methoxybenzyl isothiocyanate (3-MBITC), which was highly toxic to several insect larvae (Bartelt and Mikolajczak, 1989). The poten-

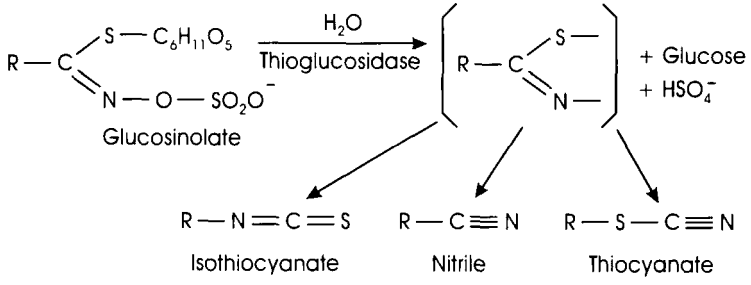

FIG. 1. Pathway and major products of glucosinolate degradation.

tial of using meadowfoam seedmeal as a soil amendment prompted us to determine if the observed reduction in weed populations were due to phytotoxins such as 3-MBITC, and if so, to identify the inhibitory compound(s).

METHODS AND MATERIALS

Gas Chromatography and Spectroscopy. Gas chromatography (GC) was performed using a Hewlett-Packard 5890 Series II gas chromatograph with a Hewlett-Packard HP3396 integrator. Columns used were fused silica DB-1 capillaries (0.25-μm film thickness, 15 m $\times$ 0.25 mm ID, J & W Scientific, Folsom, CA). Mass spectra were produced by introducing samples through a Hewlett-Packard 5890 gas chromatograph connected to a Hewlett-Packard 5971 Mass Selective Detector. ^{1}H and ^{13}C NMR spectra relative to tetramethylsilane were measured in $CDCl_3$ or D_2O at 400 and 100 MHz, respectively, with a Bruker ARX-400 spectrometer.

Extract Preparation. Hexane-extracted meadowfoam seedmeal was obtained from the Oregon Meadowfoam Growers Association, Salem, Oregon. Extracted seedmeal was first ground in a coffee grinder to a fine powder to facilitate extraction. Samples of 100 g were each extracted with 300 ml of ethyl ether, allowed to stand covered for 24 hr and filtered through folded filter paper circles (Whatman 2V). The extraction was repeated twice with additional 300 ml aliquots of ether, each time allowing the seedmeal to remain in the ether for 1 hr. The filtrates were combined and concentrated by rotoevaporation. The seedmeal was air-dried and extracted three times with absolute ethanol (300 ml for each extraction). The resulting extracts were combined and concentrated by rotoevaporation. The remaining seedmeal was extracted three times with distilled water (300 ml), the extracts combined and freeze-dried.

Chromatography of Extracts. The ethyl ether and ethanol extracts were chromatographed on a 4 $\times$ 52 cm column of Sephadex LH-20, using dichloromethane-ethanol, 1 : 1 (v/v) as the eluent. Ten fractions from the ether extract, and seven from the ethanol extract corresponding to colored bands were collected. Fractions found to be active by bioassay (see below) from both extracts were analyzed by GC. Comparison of retention time with a commercially-available standard (Aldrich), and GC-MS and NMR analysis identified the compound as (3-methoxyphenyl)acetonitrile (3-MPAN). Prominent diagnostic mass spectral ions, their relative intensities, and ^{1}H- and ^{13}C-NMR spectra for 3-MPAN are as follows: EI-MS [m/z (%)]: 147 (100), 132 (22), 117 (18), 116 (19), 107 (14), 91 (10), 77 (33). ^{1}H-NMR (400 MHz, $CDCl_3$): 3.81 (s, 3H); 4.67 (s, 2H); 6.87 (m, 3H); 7.29 (t, J = 7.9 Hz, 1H). ^{13}C-NMR (100 MHz, $CDCl_3$): 48.5 (CH_2); 55.24 (CH_3); 112.33 (CH); 113.73 (CH); 118.91 (CH); 129.96 (CH); 132.25 (C); 135.64 (C); 159.95 (C).

Glucosinolate Analysis of Seedmeal. Because unhydrolyzed glucosinolates present in the seedmeal may be further degraded to phytotoxins when in soil, glucosinolate concentrations were determined using a modified aglycone analysis procedure developed by Daxenbichler and VanEtten (1977). One hundred g of seedmeal were blended in a Waring blender with 500 ml of 30% (v/v) aqueous methanol and allowed to stand for 1 hr. The eluate was filtered through folded Whatman 2V filter circles and the marc washed with additional aliquots (~200 ml each) of aqueous methanol. The eluate volume was reduced by rotoevaporation in which all of the methanol was removed. The residue was transferred to a centrifuge tube with water to which 2 ml of a 0.5 M barium acetate/lead acetate solution was added, and the mixture was centrifuged (2500 rpm, 10 min). The supernatant was decanted (the pellet was discarded), freeze-dried, and redissolved in 15 ml of 0.05 M K-phosphate buffer, pH 7.0 to which 25 mg of thioglucosidase (Sigma) was added. Fifteen ml of CH_2Cl_2 containing 1 mg/ml butyl isothiocyanate as an internal standard was added to the buffered solution, and was shaken at 30 C for 2 hr. After shaking the organic layer was separated from the aqueous layer and analyzed by GC.

Analysis of Headspace Volatiles by Solid Phase Microextraction. Residual glucolimnanthin in the seedmeal may be further degraded to volatile phytotoxins such as 3-MPAN or 3-MBITC. Volatiles released from seedmeal that was wet to field capacity and allowed to remain moist and aerated for up to 7 days were examined using a solid phase microextraction (SPME) device (Supelco, Inc., Bellefonte, Pennsylvania). Briefly, SPME consists of a 1-cm fused silica fiber which is coated with a stationary phase, and attached to a stainless steel needle/plunger which is contained in an adjustable holder. The fiber is extended and placed in the headspace above the sample, where organic compounds adsorb to the stationary phase, reaching equilibrium in 2–30 min. After the sample is adsorbed, the fiber is withdrawn into the needle, and rapidly introduced into a gas chromatograph injector, where the adsorbed compounds are thermally desorbed onto the column (Zhang and Pawliszyn, 1993; Boyd-Boland et al., 1994). Headspace volatiles of the seedmeal were obtained by placing 100 g of seedmeal in a 600 ml beaker, and subsequently wetting the seedmeal to field capacity with distilled water. Aluminum foil was placed over the beaker, sealed, and allowed to stand for 15 min. After this time, the SPME device (which had been preconditioned immediately before by placing it into a second GC injection port not connected to the column) containing a 100 μm polydimethylsiloxane phase fiber was inserted through the foil into the headspace above the wet seedmeal, the fiber extended and allowed to adsorb volatiles for 15 min. After sample adsorption, the fiber was withdrawn and immediately injected into the GC by inserting the needle through the injector port septum and extending the fiber inside the injector insert for 0.2 min. Subsequent GC methods were the same as with liquid samples.

Seedling Radicle Elongation Bioassay. Wheat (*Triticum aestivum* L. "Cardinal") and velvetleaf (*Abutilon theophrasti* Medicus; purchased from Valley Seed Service, Fresno, California) seeds were surface disinfected with 0.5% commercial chlorine bleach for 15 min, after which they were rinsed with and then soaked in sterile distilled water for 2 hr. Seeds were wrapped in sterile paper towels saturated with water and incubated overnight in darkness at 25°C. All crude and column extracts were assayed by adding extracts to autoclaved water agar (1.0% w/v) at the rate of 1 mg extract/ml agar, after the agar had cooled to ~40°C. Fifteen ml of the agar-extract mixtures were placed in 9.0 cm plastic petri dishes, and five germinated seedlings were placed on the agar in the petri dishes. The dishes were incubated in darkness at 25°C on 45° slants, then evaluated visually for radicle growth.

Solutions of 3-MPAN, 3-MBITC and glucolimnanthin were assayed as above by adding stock solutions of the compounds in methanol (3-MPAN, 3-MBITC) or water (glucolimnanthin) to cooling water agar (the control contained methanol only). Radicle lengths (6 plates of 4 seedlings each) were measured after 48 h of incubation, and I_{50} values (the amount of each compound required to reduce radicle elongation by 50%) were determined from the intercept of 50% of the control with a best fit line of the data using regression analysis (StatView, Abacus Concepts, Berkeley, CA).

RESULTS AND DISCUSSION

Identification of the Phytotoxin. Both ethyl ether and ethanol extracts visibly inhibited velvetleaf and wheat radicle elongation, while the water extract had no effect. Fractionation of the crude extracts on the Sephadex LH-20 column gave one fraction in each extract that was inhibitory to radicle elongation in bioassays, although the active ether fraction was less inhibitory than the active ethanol fraction when tested at 1 mg extract/ml agar. Comparisons with GC retention times of a commercial standard, GC-MS and ^{1}H-, ^{13}C-NMR spectral data indicated that the inhibitory compound was 3-MPAN. The 3-MPAN peak accounted for ~97.5% of the total peak area of the active ethanol fraction (Figure 2), and greater than 90% of the active fraction from the ether extract. The remaining ~2.5% in the ethanol extract was identified as 3-hydroxy-3-methylbutanenitrile by comparison of sample mass spectra with published mass spectra (Spence and Daxenbichler, 1980). 3-MPAN is a hydrolysis product of glucolimnanthin, the major glucosinolate of *L. alba* and several other *Limnanthes* species (Miller et al., 1964; Bartelt and Mikolajczak, 1989). The 100 g of the seedmeal we tested contained 149 mg of extractable 3-MPAN. Unextracted *L. alba* seed was shown to release primarily (~90%) 3-methoxybenzyl isothiocyanate (3-MBITC) during hydrolysis, with only minor (~10%) amounts

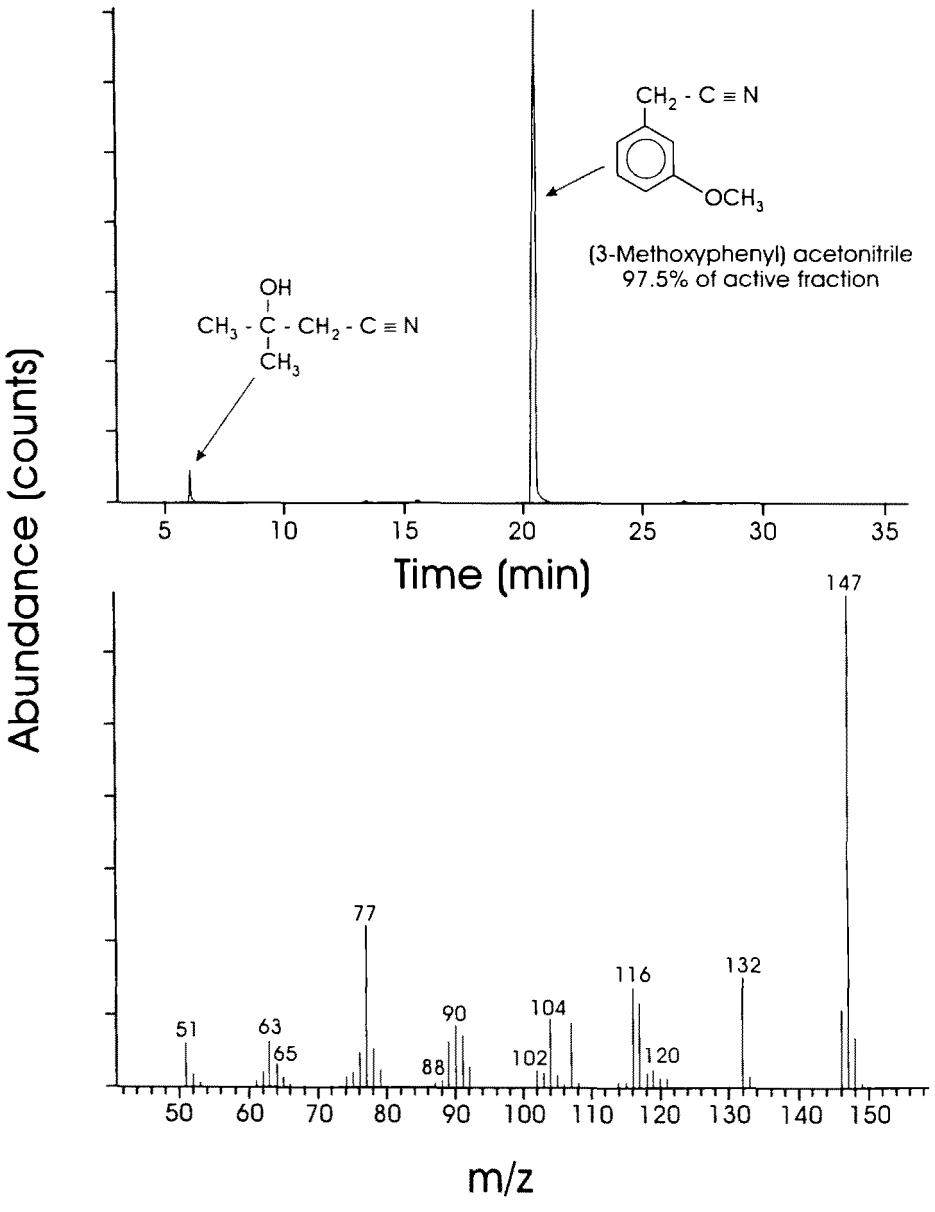

FIG. 2. Gas chromatograph of active ethanol fraction (top) and mass spectrum of 3-MPAN (bottom).

of 3-MPAN (Bartelt and Mikolajczak, 1989). 3-MBITC was not detected in either the ether or ethanol extracts. However, it is known that the hydrolysis products of glucosinolates vary with conditions of hydrolysis or prior treatment of the sample (Cole, 1976; Van Etten and Tookey, 1983), and that nitriles are produced under high temperature autolysis, as meadowfoam seed are heated to 220–230 C prior to oil extraction to inactive native thioglucosidase activity (K. D. Carlson, personal communication).

Glucosinolate Analysis. Reaction of unhydrolyzed glucosinolates in the seedmeal with exogenous thioglucosidase produced a markedly different chemical profile than with solvent extraction (Figure 3). 3-MBITC was the major (74.6%) peak, equal to 49 mg of 3-MBITC per 100 g of seedmeal, while 3-MPAN only accounted for 8.9% of the total peak area, or approximately 6 mg per 100 g of the seedmeal. 3-Hydroxy-3-methylbutanenitrile (1.9% of the total peak area), 5,5-dimethyloxazolidine-2-thione (6.4%) and an unknown (the mass spectra suggests a hydroxy-substituted 3-MPAN; 8.2%) were the other compounds detected.

Analysis of Headspace Volatiles by SPME. Relative concentrations of volatiles collected over headspace gases of wet meadowfoam seedmeal by SPME

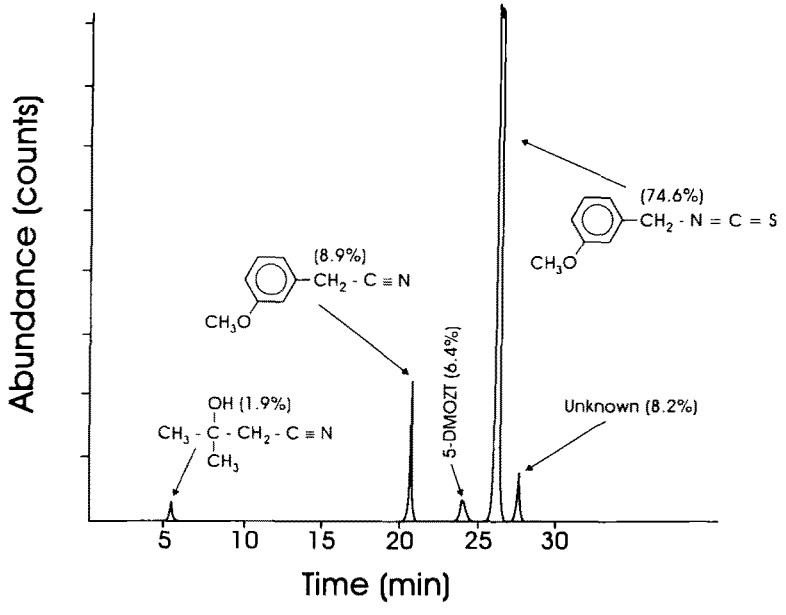

FIG. 3. Gas chromatograph of thioglucosidase-catalyzed degradation products of residual glucosinolates in seedmeal.

TABLE 1. RELATIVE CONCENTRATIONS OF VOLATILE COMPOUNDS COLLECTED BY SPME OVER HEADSPACE GASES OF MOISTENED MEADOWFOAM SEEDMEAL

Time after seedmeal moistened	% of total volatiles collected[a]		
	3-MPAN	Nonanal	3-Methoxy benzaldehyde
Initial	94.3	2.0	0.7
1 day	95.3	1.4	0.7
2 days	94.7	1.7	0.8
4 days	96.1	1.3	0.6
7 days	98.8	0.2	0.1

[a] Values represent the mean of three replicates.

are shown in Table 1. Due to the nature of SPME, quantification of compounds is difficult, so only percentages of total volatile compounds collected are shown. 3-MPAN accounted for over 94% of the total volatile profile in the freshly-moistened seedmeal, with other significant peaks identified by comparison of retention times and mass spectra of standards as nonanal (~2% of the total peak area) and 3-methoxybenzaldehyde (~0.7%). Seedmeal that was kept moist, aerated and allowed to decompose contained more 3-MPAN after seven days than freshly-moistened seedmeal, accounting for over 98% of the volatiles collected. 3-MBITC was not detected at any sampling time, even though the glucosinolate analysis indicated that significant levels of unhydrolyzed glucolimnanthin remained in the seedmeal after hexane extraction. Evidently the extraction procedure altered glucolimnanthin degradation, as Bartelt Mikolajczak (1989) had shown that intact *L. alba* seed produced primarily 3-MBITC, not 3-MPAN.

Toxicities of 3-MPAN, 3-MBITC and Glucolimnanthin. 3-MPAN, 3-MBITC and glucolimnanthin were assayed against velvetleaf and wheat seedlings to determine phytotoxic levels of each compound. The calculated I_{50}'s for 3-MPAN were 4.1×10^{-4} M and 7.4×10^{-4} for wheat and velvetleaf, respectively (Figure 4). 3-MBITC was considerably more phytotoxic, with I_{50}'s of 2.0×10^{-6} M (velvetleaf) and 5.0×10^{-6} M (wheat). 3-MBITC, although not detected in the seedmeal extracts, has been previously identified as an active component of meadowfoam seeds. Bartelt and Mikolajczak (1989) found that 3-MBITC was the primary active compound against European corn borer (*Ostrinia nubialis* Hübner) and fall armyworm (*Spodoptera frugiperda* J. E. Smith) larvae, with 3-MPAN having little or no effect on these insects. Glucolimnanthin was only slightly inhibitory to radicle elongation at the highest concentrations tested (10^{-3} and 10^{-2} M for velvetleaf and 10^{-2} M for wheat). The low toxicity of gluco-

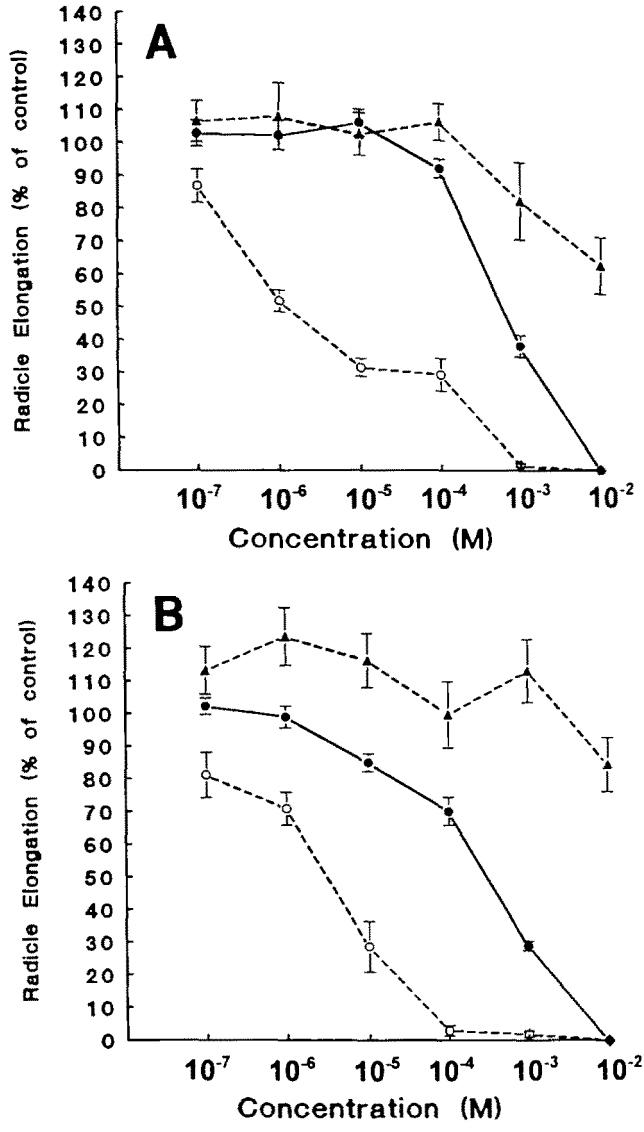

FIG. 4. Inhibition of velvetleaf (A) and wheat (B) radicle elongation by 3-MPAN (—●—), 3-MBITC (--○--), or glucolimnanthin (--△--). Bars represent ±1 SE.

limnanthin is similar to that reported by Brinker and Spencer (1993) for the glucosinolate glucoraphenin (4-methylsulphinyl-3-butenyl glucosinolate). The low phytotoxicity of glucolimnanthin was not surprising, as Bialy et al. (1990) found that glucosinolates were generally much less phytotoxic than their degradation products to wheat germination and growth.

Although our data suggest that 3-MPAN is far less phytotoxic than 3-MBITC, the relatively large amounts we found in the seedmeal help to explain the herbicidal activity we observed in our preliminary field studies. The amount of 3-MBITC produced by enzymatic degradation of the remaining glucolimnanthin represents only about one-third of the 3-MPAN directly extracted from the seedmeal. However, the much higher toxicity of 3-MBITC toward the bioassay species indicates that this compound would likely reach phytotoxic levels if the residual glucolimnanthin was primarily hydrolyzed to 3-MBITC in the soil. Although we were unable to detect any 3-MBITC in volatiles collected from moistened seedmeal, we did not examine seedmeal decomposing in soil. Brown and Morra (1995) found that defatted rapeseed meal with intact, unhydrolyzed glucosinolates severely inhibited lettuce (*Lactuca sativa* L.) seed germination while rapeseed meal in which most of the glucosinolates had been previously hydrolyzed had little or no effect on seed germination. The major volatile (>80%) released initially (0.3–2.3 hr) by moistened rapeseed meal in this study was 3-butenyl isothiocyanate, with the corresponding nitrile (3-butenyl nitrile), comprising approximately 7% of the total. Presumably during the production of the rapeseed meal, sufficiently high temperatures necessary to deactivate native thioglucosidase activity were not reached. Variability in soil type, pH (e.g., acid conditions favor the formation of nitriles) and microbial flora would likely produce different volatile profiles than we found in our study. Indeed, thioglucosidases capable of hydrolyzing glucosinolates to isothiocyanates have been isolated from bacteria and fungi (Fenwick et al., 1983). Perhaps under field conditions residual glucolimnanthin in the seedmeal would be converted to 3-MBITC by microbes, increasing its herbicidal activity. We are currently conducting more extensive field studies to determine the feasibility of using meadowfoam seedmeal as a soil amendment for weed control.

Acknowledgments—We thank The Oregon Meadowfoam Grower's Association for furnishing the seedmeal, Dr. David Weisleder for providing the NMR spectra, and Ms. Carlotta Williams for technical assistance.

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BOOK REVIEW

Analyses in Insect Ecology and Management. Larry Pedigo and Michael Zeiss. Iowa State University Press, Ames, Iowa, 1996. Hardback, ISBN 0-8138-2861-9, 168 pp. $64.95.

There is an essential suite of quantitative tools with which any applied entomologist working in pest management should have a working knowledge. This book attempts to provide an overview with working examples of many of these tools. The authors suggest that the book is intended as a primer that can be used in an autotutorial mode by students or professionals or as a laboratory manual for graduate-level courses in insect ecology or pest management. The book is organized into eight short chapters and is accompanied by a diskette of DOS computer programs (ENSTAT) that complement many of the lessons. Each chapter concludes with sample problems to exemplify the topic area covered, a series of questions, and a bibliography of selected papers. An answer key is available free of charge from the publisher. There is no index.

The first chapter is a short introduction to using the statistical portion of ENSTAT along with several small data sets for practicing use of the software. The software can perform simple one and two-way analysis of variance, chi-square analysis and simple linear regression. The program would be useful to undergraduate or beginning graduate students with little experience in statistical analysis. Most professionals and upper level graduate students will find the program's capabilities limited and would be better off learning to use one of the more widely available statistical packages. Data entry is clumsy and the user cannot import existing data files nor export data sets for further analyses.

Chapter 2 provides general coverage of the spatial distribution of insect populations. Some of the more common theoretical distributions such as the Poisson, negative binomial, and binomial distribution are explained and exemplified. Taylor's power law is covered briefly, but other common methods for empirically describing dispersion, for example the patchiness regression based on Lloyd's mean crowding index, are not mentioned. ENSTAT programs are provided to determine goodness of fit of field data to Poisson and negative binomial distributions.

Chapters 3 and 4 address the development of sampling plans. The third chapter covers basic development of sequential sampling plans for pest management application based on Wald's methods. These sequential plans basically allow a given pest population to be classified as either above or below a predetermined threshold level. They are typically quite efficient because few sam-

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ples are needed to classify low or high population densities. Standard formulae are presented for calculating sequential stop lines based around the binomial, negative binomial, Poisson, and normal distributions. The software includes spreadsheet-based programs for calculating sequential stop lines for all but the normal distribution, and for estimating average sample number and error rates as a function of pest density. The chapter concludes with a reprint of a journal article exemplifying the development of a sequential sampling plan for a soybean pest. The fourth chapter discusses a specialized use of sequential sampling (time-sequential) that attempts to classify whether a pest population is increasing or decreasing over time. Much of the concept and detail of the method is covered in a reprinted article by the first author. Again, ENSTAT contains a module for calculating stop lines.

The degree-day model for predicting temperature-dependent development of insects is the subject of Chapter 5. Coverage here is elementary and the reader may want to consult references in the bibliography and software for greater detail. For example, there is no discussion of the sinewave method or of methods that use both a lower and upper development threshold. The software for calculating and accumulating degree-days contains a few bugs, but does permit the user to specify upper developmental thresholds and provides output using the rectangular, triangular, and sinewave method.

The next two chapters cover the development and analysis of life tables. The text section is brief, with most of the detail provided in reprints of all, or parts of, several classic references by Harcourt, Ives, and Southwood. Good example data sets are provided for the practice construction and analysis of age-specific life tables. The authors also provide a fairly detailed set of step by step instructions for doing k-factor and key-factor analyses, techniques that attempt to identify causative factors in the population dynamics of a species over time and/or multiple sites. The text and bibliography discuss density-dependence, but do not address the continuing controversy over the validity of various techniques for measuring density-dependence. ENSTAT contains a module for life table construction.

The final chapter covers the concept and develompent of economic injury levels (EIL), one of the most fundamental components of modern IPM. The book focuses on the EIL model of Pedigo and provides step by step instructions on how to calculate an EIL for a specific soybean pest. Again, the chapter concludes with a reprint of a journal article. The authors do not discuss other EIL models nor present methods for determining the economic threshold, the actual pest density at which suppressive action should be implemented.

Analyses in Insect Ecology and Management is well written, and printed in fairly large type in single columns making it easy to read. Based on the subject matter covered, the level of detail, and the presentation format, the book would be most useful as a supplemental text for upper undergraduate or lower

level graduate laboratory courses in insect pest management. Cost may be prohibitive, particularly if another book is required for the lecture portion of the course. Professionals wanting a good reference to some quantitative techniques would probably be a bit frustrated by the lack of depth, and those using the book in an autotutorial mode would definitely want to obtain the answer key. I am not a big fan of books that reprint original research articles that could be easily obtained elsewhere, but the approach does provide for self-contained lessons. The ENSTAT software has a few bugs, but is useful in furthering the educational value of the text. Most graduate students and professionals will quickly outgrow the programs.

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PHENYLPROPENOID PHENOLICS IN SWEETBAY MAGNOLIA AS CHEMICAL DETERMINANTS OF HOST USE IN SATURNIID SILKMOTHS (*Callosamia*)

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Abstract—Host plant chemistry can play an important role in determining the evolution of host use patterns in herbivorous insects by influencing host selection, consumption, and assimilation of foliage. We used a comparative approach to test the hypothesis that specialist herbivores of sweetbay magnolia (*Magnolia virginiana*) possess adaptations that allow them to overcome chemical deterrents or toxins that prevent herbivory by unadapted herbivores. The three silkmoth species in the genus *Callosamia* can be collectively regarded as specialists on magnoliaceous hosts; however, only the monophagous *C. securifera* is able to complete development on sweetbay magnolia, its natural host. In laboratory assays with intact foliage, both *C. angulifera* and the polyphagous *C. promethea* fed readily on sweetbay but were unable to survive past the third instar. Two neolignan compounds, magnolol and a biphenyl ether, were found to reduce neonate growth and survival of unadapted herbivore species when painted on acceptable host leaves at concentrations similar to those found in sweetbay foliage. Both compounds significantly reduced neonate growth of *C. angulifera* and *C. promethea* but had no effect on the sweetbay specialist, *C. securifera*, indicating that the latter species possesses the unique ability in the genus to tolerate, metabolize, or otherwise circumvent the phytochemical defenses of this host.

Key Words—Phenolics, sweetbay magnolia, silkmoths, Saturniidae, phenylpropenoids, *Magnolia virginiana*, *Callosamia*, host plant chemistry.

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INTRODUCTION

Early in the development of plant–herbivore theory, host plant chemistry was recognized as an important determinant of host use patterns of phytophagous insects on both an ecological and evolutionary scale (Fraenkel, 1959; Ehrlich and Raven, 1964). More recently, enthusiasm for the importance of plant chemistry in the evolution of insect–plant interactions has been tempered by the recognition that natural enemies, pathogens, and other ecological restrictions may exert equally powerful and potentially conflicting, pressures on host use patterns (Lawton and McNeill, 1979; Price et al., 1980; 1986; Scriber and Lederhouse, 1992; Bernays and Graham, 1988). Nonetheless, in many cases where herbivore phylogenies and host use patterns can be reasonably well defined, there is little doubt that host plant characteristics do profoundly influence the evolution and diversification of some phytophagous taxa (Benson et al., 1975; Mitter and Brooks, 1983; Farrell and Mitter, 1990). Plant allelochemicals, in particular, appear to have played a central role in the evolution of host patterns in *Papilio* butterflies (Feeny et al., 1983; Feeny, 1991; Miller, 1987; Scriber, 1988), leaf beetles (Futuyma and McCafferty, 1990), *Euphrydras* checkerspot butterflies (Bowers, 1988), and *Heliconius* butterflies (Spencer, 1988). Thus, the concept of toxic and/or deterrent compounds acting as chemical constraints or barriers to host use remains a prominent feature in discussions of phylogenetic patterns of host use (Miller, 1987; Berenbaum et al., 1986; Feeny, 1991; Nitao et al., 1992; Nitao, 1995; Scriber et al., 1989).

The presence of robust chemical defenses in plants can present substantive evolutionary hurdles for host range expansion or shifts to novel hosts. Such alterations in host use patterns may require behavioral changes, such as those involved in oviposition and feeding, as well as physiological or metabolic changes that allow the herbivore to utilize efficiently the particular nutritional and chemical characteristics of the new host. Thus, specific allelochemicals or nutritional characteristics may act as chemical barriers in both ecological and evolutionary patterns of resource use of herbivores lacking these adaptations (Berenbaum et al. 1986, 1991). Demonstrations of the deterrent or toxic effects of phytochemicals on individual herbivore species reveal little information regarding the potential evolutionary significance of these compounds. However, comparative measures of the effects of plant compounds on closely related herbivores have proven to be valuable for identifying metabolic or detoxicative constraints on host use (Erickson and Feeny, 1974; Blau and Feeny, 1978; Berenbaum, 1978, 1981; Lindroth et al., 1986, 1988; Scriber et al., 1989; Nitao et al. 1992).

The wild silkmoths in the genus *Callosamia* (Saturniidae) represent a good model system for investigating the role of plant chemistry on the evolution of host use patterns. Collectively, this small group of saturniids can be

considered specialists on magnoliaceous hosts, yet the three species in the genus differ dramatically in both breadth of diet and their ability to survive on specific host plants. *C. securifera* is a specialist, feeding exclusively on sweetbay magnolia (*Magnolia virginiana*) in the southeastern United States. In contrast, *C. angulifera* is oligophagous and feeds primarily on tuliptree (*Liriodendron tulipifera*: Magnoliaceae) and occasionally spicebush (*Lindera benzoin*: Lauraceae). The third species, *C. promethea*, is highly polyphagous and feeds on deciduous trees in over five different plant families, including black cherry (Rosaceae), sassafras and spicebush (Lauraceae), tuliptree (Magnoliaceae), ash and lilac (Oleaceae), buttonbush (Rubiaceae), and others (Ferguson, 1972; Peigler, 1976; Stone 1991). Although sweetbay occurs within the geographic distributions of the two *Callosamia* species with broader feeding habits, neither *C. promethea* (with the exception of some populations in Maryland; H. Pavulaan, personal communication) nor *C. angulifera* use it as a host. Laboratory observations of early instar feeding behavior led to the hypothesis that sweetbay foliage is toxic to unadapted herbivores.

This study was undertaken to confirm and characterize the phytochemical basis for the differential performance of the three wild silkmoths on sweetbay magnolia in an effort to understand the evolution and physiological basis of host specialization within the *Callosamia* lineage.

METHODS AND MATERIALS

Larval Performance on Fresh Leaves. The relative suitabilities of tuliptree, sassafras, and sweetbay as hosts for each *Callosamia* species was evaluated in June 1989 using measures of neonate growth rates and survival on intact foliage. Newly eclosed larvae were weighed and reared individually in Petri dishes on detached leaves kept fresh with water-filled plastic vials with rubber caps. The relative growth rate of each larva was calculated as RGR = [ln (final mass) − ln (initial mass)/2 days]. First-instar survival was determined by the number of larvae that successfully completed the molt to the second stadium. Tuliptree and sassafras foliage used in the feeding trial was collected in Ingham County, Michigan; sweetbay foliage was from potted trees (originally obtained from Herren Nursery, Florida Division of Forestry) maintained in a greenhouse at Michigan State University for several years. Insects used in the assays on host foliage were from five wild-collected *C. securifera* females (Highlands County, Florida), one *C. promethea* (Wisconsin) and one *C. angulifera* female (Lebanon County, Pennsylvania). While better representation of populations would have been desirable, additional females were not available. Larvae were distributed evenly across the three hosts (25–30 larvae per host) and reared at 24°C and a 18:6 light-dark photoperiod. Because the *C. promethea* trial was conducted 10

days earlier than the other two species, each species trial was analyzed separately. Significance of differences in growth rates on sassafras, tuliptree, and sweetbay were tested by one-way analysis of variance followed by Tukey's multiple range test.

Extraction and Bioassay of Crude Extracts from M. virginiana *Leaves.* Fresh sweetbay leaves (mixture of young and old) were removed from trees, freeze-dried, and milled. The powdered leaf material (435 g) was sequentially extracted at room temperature with hexane (6 liters), ethyl acetate (6 liters), and methanol (2 liters). Removal of solvent *in vacuo* yielded 36.9 g, 16.9 g, and 54.34 g of residue, respectively.

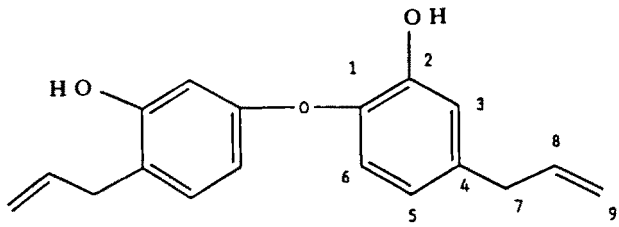
Because *Callosamia* larvae do not accept artificial diet, extracts were painted on fresh leaves of tulip tree, which is an acceptable host for both *C. promethea* and *C. angulifera*. Extracts were dissolved in an appropriate solvent (hexane or methanol) and applied to the top surface of detached leaves with a syringe. The amount applied to each leaf was adjusted for the fresh weight of that leaf to approximate concentrations in fresh *M. virginiana* leaves (grams of extract per gram of fresh leaf). Control leaves were treated with an equivalent volume of the appropriate solvent. Freshly treated leaves were added after 24 hr or as needed.

Newly eclosed larvae were weighed and allowed to feed individually on treated leaves in plastic cups for 72 hr at 24°C and a 18:6 light–dark photoperiod, after which they were reweighed and 48 hr relative growth rate calculated as described above. A 1-cm layer of moistened plaster of Paris in the bottom of each cup served to maintain mositure and leaf turgor during the experiment. Larvae used in bioassays were obtained from field-collected or first-generation laboratory females (*C. promethea* were from Cass County and Otsego County, Michigan; *C. angulifera* were wild females from Greensville County, Virginia, and Florence County, South Carolina). Due to the difficulty of coordinating egg hatch of female moths to obtain similar-aged neonates, only two families of each species were represented in each assay. Fifteen neonates were used in each treatment. Tukey's HSD multiple comparison test was used to detect differences ($P < 0.05$) in growth rate of each species on the extracts.

Isolation and Bioassay of Individual Compounds from Active Fraction. The hexane fraction (12.78 g) of sweetbay extract was found to be the most active against neonates, so its constituents were further separated using centrifugal partition chromatography (Sanki Engineering, Kyoto) using the lower phase of hexane–acetonitrile–ethyl acetate–water (40:35:25:5) as the mobile phase at a flow rate of 3.2–3.8 ml/min at 900 rpm. Three major compounds were purified by flash column (Sigma silica 230–400 mesh, 60 Å eluted with hexane–ether 4:1) and preparatory thin layer chromatography (silica gel, 2000 μm tapered Uniplate, Analtech, Newark, Delaware, developed twice in hexane–ether 4:1). The final purification yielded three neolignan compounds: 4,4′-diallyl-2,3′-di-

hydroxybiphenyl ether (147.8 mg), 5,5′-diallyl-2,2′-dihydroxybiphenyl (100 mg) and 3,5′-diallyl-2′-hydroxy-4-methoxybiphenyl (207.6 mg). These compounds were identified from NMR, MS, UV, and IR spectra (Nitao et al., 1991). Structures for these compounds are shown in Figure 1 and hereafter they are referred to as the biphenyl ether (**1**), magnolol (**2**), and methoxyhonokiol (**3**), respectively.

The biphenyl ether, magnolol, and methoxyhonokiol were bioassayed at 0.03%, 0.125% and 0.5% (grams of compound per gram fresh tuliptree leaf) against *C. promethea* neonates using the leaf-painting technique described previously. Natural concentrations of these compounds in sweetbay foliage were estimated to be 0.46, 0.31, and 0.64% fresh wt (FW), respectively, from our extraction procedure, but dose–response curves are more informative than single-dose assays of plant secondary metabolites because concentrations of com-



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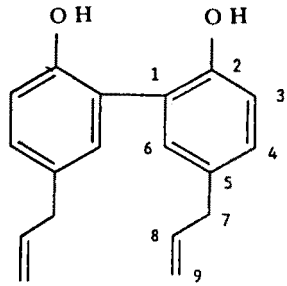

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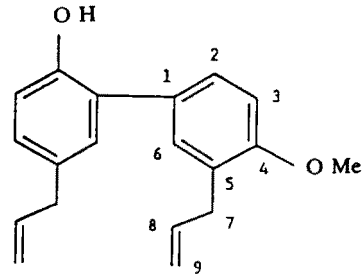

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FIG. 1. Structures of *Magnolia virginiana* neolignans active against larvae of unadapted *Callosamia* silkmoth species. **1** = 4,4′-diallyl-2′,3′-dihydroxybiphenyl ether; **2** = magnolol; **3** = 4-methoxyhonokiol.

pounds in plants in the field may vary considerably. Assays were replicated three times using *C. promethea* collected from distantly separated geographic locations (Montgomery County, Maryland; Lebanon County, Pennsylvania; several locations in Wisconsin and Michigan), with 6–10 larvae represented per concentration per assay. Because *C. angulifera* were difficult to obtain, only magnolol was tested against this species. (*C. angulifera* larvae were from wild females collected in Cass County, Michigan; Greensville County, Virginia; and Mississippi). In the *C. angulifera* assays, magnolol was assayed at five doses rather than three (0.1, 0.2, 0.3, 0.4, and 0.5% grams of compound per gram fresh leaf). As an additional check of the leaf-painting technique, the responses of *C. securifera* neonates (several families from Highlands County, Florida) to magnolol and the biphenyl ether were assayed at 0.1, 0.3, and 0.5%. Larval response to host chemicals was measured in terms of 48-hr relative growth rate. Data from the replicate assays were pooled, and a polynomial regression model used to test for linear and quadratic dose-dependent effects.

RESULTS

Larval Performance on Intact Host Plants. Relative growth rate and first-instar survival of both *C. promethea* and *C. angulifera* were considerably reduced on sweetbay compared to the two acceptable hosts (Figure 2A and B), whereas *C. securifera* performed as well on sweetbay as on sassafras and significantly better than on tuliptree. First-instar survival for *C. promethea* and *C. angulifera* was less than 30% on sweetbay but ranged from 90 to 100% on acceptable hosts. *C. securifera* survival was greater than 70% on sweetbay compared to 62% on sassafras and 85% on tuliptree. The patterns of short-term larval growth rates corresponded closely with larval survival, with the exception of *C. securifera*'s response to tuliptree. On this host, growth was significantly lower than on sweetbay and sassafras, although survival remained high. No conspicuous differences in larval feeding rate were observed, suggesting that the reduced growth and survival were not entirely due to strong antifeedant effects.

Separation and Bioassay of Crude Extract. Of the three major fractions, the hexane extract produced the greatest reduction in larval growth rate (one-way ANOVA, *C. promethea*: $F = 7.86$; $df = 1, 3$; $P < 0.001$; *C. angulifera*: $F = 9.79$; $df = 1, 3$; $P < 0.001$). *C. promethea* and *C. angulifera* responded similarly to all three fractions with a significant reduction of growth on leaves treated with the hexane extract compared to controls [Figure 3A: *C. promethea* (mean ± SE) 0.26 ± 0.04 mg/mg/day; *C. angulifera* 0.34 ± 0.03 mg/mg/day]. *C. angulifera* growth on leaves treated with the ethyl acetate fraction was also significantly lower than on control leaves (0.45 ± 0.03 mg/mg/day). Percent survival of both species was also lowest on leaves treated with the hexane

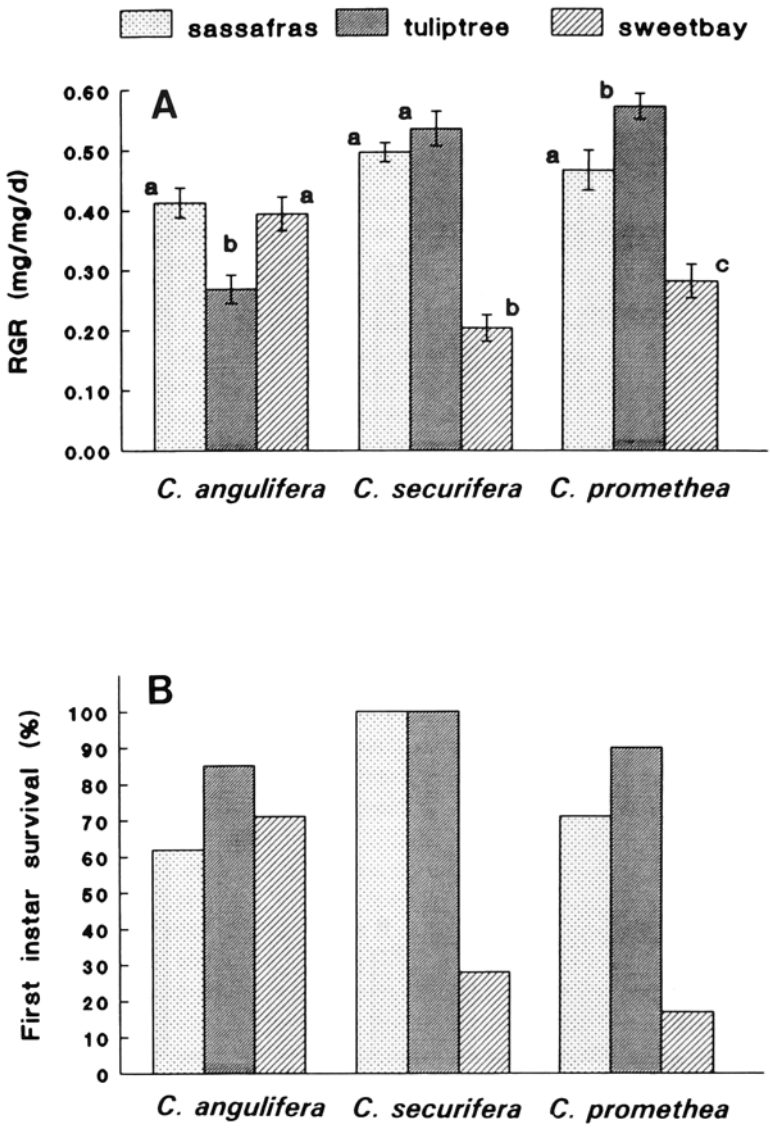

FIG. 2. Relative growth rate and first-instar survival of *C. securifera*, *C. angulifera*, and *C. promethea* on sassafras (*Sassafras albidum*), tuliptree (*Liriodendron tulipifera*), and sweetbay magnolia (*Magnolia virginiana*). Different letters denote significant differences in growth among host plants for each species. $N = 25$–30 larvae.

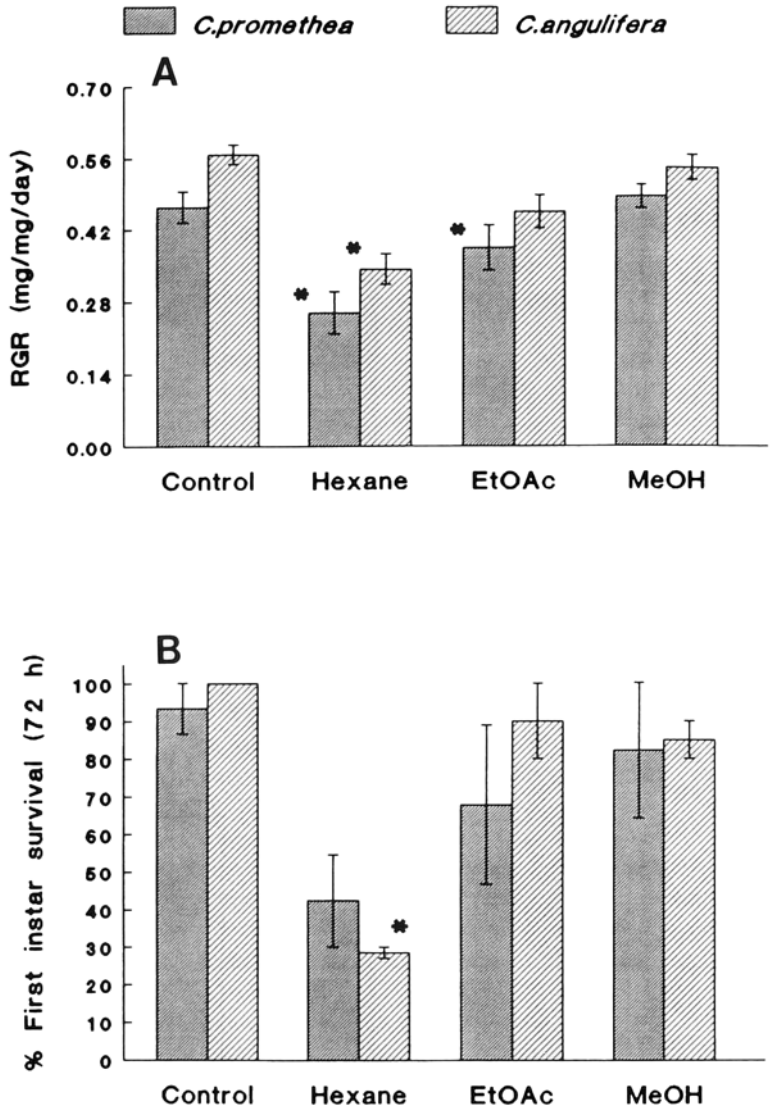

FIG. 3. Relative growth rates and survival of neonate larvae of the two unadapted silkmoth species, *C. promethea* and *C. angulifera*, on tuliptree leaves painted with extracts from *Magnolia virginiana* foliage. Bars represent the means ± SE for 15 larvae from at least two geographically separated populations. Asterisks denote treatments that differ significantly from the control.

fraction [Figure 3B: *C. promethea* (mean ± SE) 42.3 ± 12.3 mg/mg/day; *C. angulifera* 28.5 ± 1.5 mg/mg/day].

Isolation and Bioassay of Individual Compounds from Active Fraction. Magnolol and the biphenyl ether were active against larvae of the unadapted species (Figure 4A and B) at concentrations approximating those found in fresh leaf material (0.31 and 0.46% FW for magnolol and the biphenyl ether, respectively). Relative growth rates of *C. promethea* larvae were significantly decreased by both magnolol (linear response: $F = 14.31$; $df = 1,75$; $P = 0.003$) and the biphenyl ether (linear response: $F = 18.89$; $df = 1,74$; $P = 0.001$). *C. angulifera* neonate growth was also significantly decreased by magnolol (linear response: $F = 18.01$; $df = 1,149$; $P = 0.001$) in a dose-dependent fashion. As expected, *C. securifera* larvae were unaffected by magnolol and the biphenyl ether across all doses. Methoxyhonokiol (Figure 4C) had no significant effect on *C. promethea* performance (linear response: $F = 0.64$; $df = 1,30$; $P = 0.430$).

DISCUSSION

Our results demonstrate that foliar chemistry may have a strong influence on the performance of *Callosamia* species on sweetbay. The poor growth and survival of *C. promethea* and *C. angulifera* on this host could be explained largely by the activity of two neolignan compounds, magnolol and the biphenyl ether, isolated from the foliage. When assayed individually at doses similar to those found in fresh leaf material, each compound reduced larval growth to levels similar to those observed on intact foliage. Potentially, the presence of either or both of these compounds could block host shifts by herbivores that lack specific metabolic mechanisms to cope with them, even within lineages that are adapted to utilize Magnoliaceous hosts, such as *Callosamia*. In this taxon, the use of sweetbay apparently requires specialized metabolic adaptations to allelochemicals other than those that accompany the ability to feed on tuliptree or those associated with a highly polyphagous feeding habit.

Host plant chemistry can affect larval performance through antifeedancy or other behavioral effects, as well as by toxic or antinutritive means (Bernays and Chapman, 1987; Feeny, 1991). Although distinguishing toxicity from antifeedancy in insect bioassays is often problematic, we saw no evidence that the compounds we isolated were acting as strong antifeedants in our assays, as larvae appeared to feed equally on treated and control leaves. This indicates that the painting of the extracts on the leaves was a legitimate technique to use in this instance. Moreover, many lignans have been reported to have biological activity (MacRae and Towers, 1984; Bernard et al. 1989; Faure et al., 1990), and both magnolol and the biphenyl ether are known to possess a broad spectrum

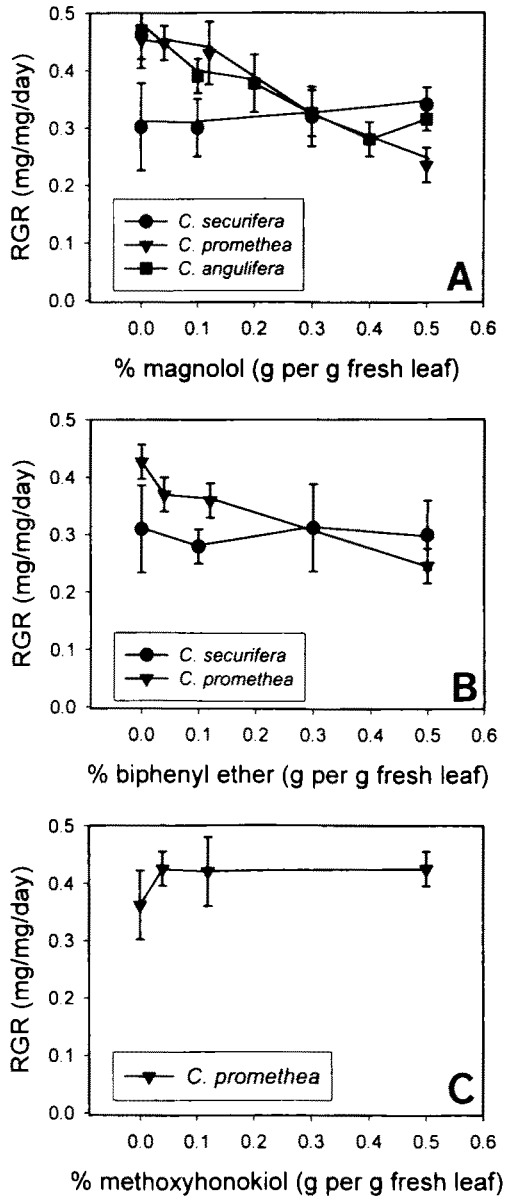

FIG. 4. Concentration-dependent effects of magnolol on the three *Callosamia* species. The relative growth rates of *C. promethea* and *C. angulifera* decreased significantly with increasing magnolol concentrations ($P < 0.003$; $P < 0.001$), whereas *C. securifera* was unaffected. Each value represents the mean ± SE of three replicate assays with 6–10 larvae per replicate.

of antimicrobial and insecticidal activity against other organisms, including other lepidopteran larvae (Clark et al., 1981; Nitao et al., 1991, 1992).

In the course of our laboratory experiments, *C. promethea* populations from many geographic locations (Wisconsin, Michigan, Pennsylvania, Maryland) were assayed, and no evidence of the ability to utilize sweetbay as a host was found, with the exception of a population near Rockville, Maryland, which were utilizing sweetbay in the wild. Bioassays of insects from these populations indicated that they are no less susceptible to the deleterious effects of magnolol and the biphenyl ether than other *C. promethea* populations (0.5% biphenyl ether reduced RGR from 0.41 mg/mg/day on control leaves to 0.19 mg/mg/day; 0.5% magnolol reduced RGR from 0.37 on control leaves to 0.21 mg/mg/day). A more likely explanation is that sweetbay growing in Maryland contains lower concentrations of neolignans than trees in other geographic regions. Spatial, temporal, intra- and interplant variation in foliar chemistry is a common occurrence in natural plant populations, resulting from both genetic and environmental factors. Sweetbay can be found growing in a variety of habitats across its range, exhibiting considerable plasticity in deciduousness, growth habit, and leaf shape. There may be geographic differences in leaf chemistry between the northern and southern populations as well. The negative effects of magnolol and the biphenyl ether in bioassays were linearly concentration dependent; thus it seems reasonable to expect that foliage containing lower concentrations will have less of an impact on larval performance.

It would appear that *C. securifera* possesses unique biochemical and/or physiological traits that afford larvae a greater tolerance of the toxic phytochemistry of its preferred host. However, it is not clear whether these traits are derived characteristics that evolved in association with increased feeding specialization of this species or whether they represent a more primitive character state that has subsequently been lost in other species of *Callosamia*. Attributing evolutionary patterns of host single causal determinants, such as current host chemistry, must be done with the great caution (Barbosa, 1988; Rausher, 1988; Thompson, 1988). Without accurate knowledge of the phylogenetic relationship of the three *Callosamia* taxa, the evolutionary significance of current ecological responses to magnolol and the biphenyl ether is difficult. We have little information about ancestral and derived traits in *Callosamia*, and estimates of genetic distance from allozyme electrophoresis (Johnson et al., 1996) indicate that the three species are essentially equidistant from each other. This is in contrast to our initial assumption that *C. securifera* was more closely related to *C. angulifera*, based on its historical placement as a subspecies of the latter by many taxonomists until the temporal reproductive isolating mechanism was described (Brown, 1972; Ferguson, 1972). Gene flow between the three species is prevented by diurnal separation of mating times: *C. securifera* females call in late morning, *C. promethea* in late afternoon, and *C. angulifera* mate after dark.

Although the physiological mechanisms controlling mating time are unknown, it would not be difficult to imagine that a relatively simple change may have produced more or less simultaneous isolation of the three species. It is conceivable that the inability of unadapated *Callosamia* to survive on sweetbay contributed to selection for mechanisms preventing hybridization between sweetbay feeding populations and those feeding on other hosts.

Compounds other than neolignans may also be involved in the chemical defenses of sweetbay magnolia, as a variety of biologically active compounds, such as phenolics, quaternary alkaloids, and sesquiterpene lactones have been isolated from other *Magnolia* and *Liriodendron* species (Doskotch and Flom, 1972; Rao and Juneau, 1975; Rao and Davis, 1982; El-Feraly and Yee-Ming, 1978; Barbosa et al., 1990). However, the biphenylpropanoids have been implicated as important determinants of the differential host use patterns of another group of Magnoliaceae and Lauraceae specialists, the *troilus* and *glaucus* lineages of the *Papilio* swallowtail butterflies (Nitao et al. 1992; Nitao 1995). The unsuitability of *M. virginiana* to *Papilio palamedes* and *P. troilus* appears to be linked to these neolignans, particularly magnolol, which reduces survival of neonates of both of these species but has no effect on *P. glaucus*, which utilizes the plant as a host in the southern part of its range. Interestingly, *P. troilus* is able to survive on leaves painted with the biphenyl ether, perhaps because similar chemicals in its lauraceous hosts (primarily sassafras and spicebush) endow it with behavioral or physiological preadaptations for biphenyl ether compounds (Nitao et al., 1992). This does not appear to be the case for *C. promethea*, which frequently feeds on the same Lauraceous hosts but clearly does not possess a similar tolerance for biphenyl ether compounds.

A better understanding of the specific biochemical/physiological adaptations of *C. securifera* to magnolol and the biphenyl ether and of the phylogenetic relationship of this species within the genus should improve our ability to evaluate the mechanisms and possible physiological trade-offs involved in the evolution of host plant specialization in the group.

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DISRUPTION OF MALE ORIENTAL FRUIT MOTH TO CALLING FEMALES IN A WIND TUNNEL BY DIFFERENT CONCENTRATIONS OF SYNTHETIC PHEROMONE

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Abstract—Disruption of male Oriental fruit moth orientation to calling females was studied in a wind tunnel by surrounding calling female moths with septa loaded with synthetic pheromone. At the lowest loadings, 0.01 and 0.1 μg, which produced release rates well below those of calling females, some males flew to septa instead of the females. At loadings of 1 and 10 μg, which produced release rates close to those of a calling female, more than half the males flew to septa instead of the females, but there was little evidence of habituation at any of these loadings. At higher loadings, 100 and 1000 μg, upwind flight of males was arrested, and many males remained inactive, indicating habituation. Preexposure of the males for 3 hr to ambient pheromone concentrations in the tunnel had no significant effect on numbers of disrupted males. However, at the 1000-μg loading, most of the males that had been preexposed to the synthetic pheromone remained inactive. This may indicate a higher level of habituation than among males that had not been preexposed, most of which flew, although they subsequently showed flight arrestment. Levels of disruption were similar to those found for the spruce budworm in comparable experiments. In both species, less than 1% of the males were able to locate females when time-averaged concentrations of synthetic pheromone were above 20 ng/m^3. However, levels of inactivity and flight arrestment were higher among male Oriental fruit moths than among male spruce budworms, which may explain why Oriental fruit moths are more susceptible to disruption than are spruce budworms.

Key Words—Oriental fruit moth, sex pheromone, mating disruption, wind-tunnel tests.

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INTRODUCTION

Synthetic pheromones are being used for the operational control of several lepidopterous pests (Cardé and Minks, 1995; and review in Ridgway et al., 1990). However, the exact manner by which the synthetic pheromone causes disruption is not known, which limits our ability to refine blends, dosages, application rates, and optimum types of formulation.

Wind tunnels provide a convenient method of examining insect behavior in response to pheromones under controlled conditions. In wind-tunnel experiments Sanders (1982, 1985, 1995, 1996) simulated field disruption of the spruce budworm [*Choristoneura fumiferana* (Clem.)], by surrounding calling female moths with dispensers of synthetic pheromone and then measuring the ability of male moths to locate the females. When the dispensers released synthetic pheromone at, or slightly above, the same rate as a calling female, a significant number of males flew to the synthetic sources instead of the females (false-trail following), but when males were left in the tunnel for a few hours they remained active and eventually located the females. This suggests that false-trail following by itself would not have prevented mating and that some other mechanism, such as habituation, is necessary to prevent males that have followed false trails from eventually locating the females. However, there was no evidence of habituation until release rates of synthetic pheromone from the disruptant dispensers were one or two orders of magnitude greater than release rates of natural pheromone by calling females (Sanders, 1985, 1995, 1996). This created a time-averaged atmospheric concentration of approximately 20 ng/m^3, which suggests that concentrations of synthetic pheromone should be maintained at least this high in the field to ensure effective mating disruption.

Mating disruption trials against the spruce budworm have met with limited success (Sanders and Seabrook, 1982; Seabrook and Kipp, 1986), which makes it difficult to compare wind-tunnel results with field results. Available evidence indicates that mating disruption becomes more effective the closer the synthetic blend is to the natural, female-emitted blend (Minks and Cardé, 1988). In the case of the spruce budworm, it is probable that the identified pheromone, a 95:5 blend of (*E*)-:(*Z*)-11-tetradecenal (Sanders and Weatherston, 1976; Silk et al., 1980), is incomplete (Alford et al., 1983; Sanders, 1984). Therefore, we have turned for comparisons to the Oriental fruit moth, *Cydia molesta* (Busck), a species that has been thoroughly researched, has a well-identified pheromone, and is successfully controlled by mating disruption in many parts of the world (Rothschild and Vickers, 1991). The pheromone was identified by Cardé et al. (1979) as a four component blend: (*Z*)-8-dodecenyl acetate, (*E*)-8-dodecenyl acetate, (*Z*)-8-dodecen-1-ol, and dodecanol. Numerous experiments have shown that a three-component blend, lacking the dodecanol, at the same concentrations as those released by calling virgin moths, elicits the full suite of mating behaviors

and that all three components in the appropriate ratios are necessary for optimum responses (Baker and Cardé, 1979a; Baker et al., 1981; Linn et al., 1986, 1987). Charlton and Cardé (1980) found that septa loaded with 100 μg of the three-component blend outperformed three caged virgin females, and Rothschild (1974, 1975) found that virgin female moths outcompeted both closed polyethylene caps loaded with 100 μg and polyethylene microcapillary tubes loaded with 50 mg of (*Z*)-8-dodecenyl acetate but that contained lower than optimum amounts of the other two components. No direct comparisons have been made between the potency of virgin female Oriental fruit moths and septa loaded with a range of concentrations of the synthetic pheromone. However, Witzgall (1996) has presented evidence from wind-tunnel observations that responses to females differ from responses to the synthetic blend, which suggests that the synthetic blend is not the exact equal of the female-produced pheromone. With this in mind, evaluations of the effectiveness of mating disruption should be carried out with calling female moths, and not just synthetic pheromone sources.

In an effort to further our understanding of why mating disruption has been limited in its success against the spruce budworm and what the mechanisms of disruption are in general among the Lepidoptera, we carried out experiments with the Oriental fruit moth in our wind tunnel. We compare the results with previous work with the spruce budworm (Sanders, 1996), which determined the ability of males to locate caged virgin female moths that were surrounded by dispensers emitting synthetic pheromone.

METHODS AND MATERIALS

Insects. The insects were provided by Dr. R. M. Trimble and staff of the Vineland Research Station of Agriculture and Agri-food Canada, Vineland Station, Ontario, Canada, from laboratory rearing stock established in the early 1970s with insects obtained from the Niagara peninsula (Trimble, personal communication). Larvae were reared on green thinning apples at the Vineland Research Station following the techniques described by Pree (1985). The mature larvae were provided with strips of corrugated cardboard that the larvae enter when ready to pupate. The cardboard strips containing larvae and pupae were then sent in insulated containers to the Sault Ste. Marie laboratory of the Canadian Forest Service. Here the pupae were sexed, and the male and female pupae were placed in separate cages in separate rooms, each maintained at 21°C and 70% relative humidity. Moths were collected daily and placed in 30-cm × 20-cm × 30-cm-high cages to await the experiments. Both male and female moths were kept on 17 L:7 D cycles. For the males scotophase was 20:30–03:30 hr. For the females scotophase was advanced to 15:00–22:00 hr to program them to be actively calling while the experiments were carried out (Baker

and Cardé, 1979b; Lacey and Sanders, 1992). The cages were sprayed with a water mist each day to provide drinking water for the moths.

Chemicals. Rubber septa (A. H. Thomas, Philadelphia, Pennsylvania; catalog No. 1780J07) that had previously been extracted with ethanol by the technique of Steck et al. (1979) were used to dispense the disruptant chemicals. Synthetic Oriental fruit moth pheromone was obtained from Bedoukian Research Inc. (Danbury Connecticut), identified as: "product P900, Bedoukian Oriental fruit moth Technical Pheromone, 93% (*Z*)-8-dodecenyl acetate, 6% (*E*)-8-dodecenyl acetate and 1% (*Z*)-8-dodecenol." A stock solution of 1 mg/ml in cyclohexane was then prepared, and this was serially diluted to provide solutions containing the appropriate quantities of the synthetic pheromone. Aliquots of 100 μl of these solutions were pipetted into the rubber septa to provide the required loadings.

Release rates of the synthetic pheromone from these septa can be calculated using the formulae:

$$R = M * 1/t^{1/2} * \log_n 2$$

and

$$M = M_o e^{(-t * 1/t^{1/2} * \log_n 2)}$$

(Butler and McDonough, 1979) where R is the release rate, M_o is the amount of pheromone loaded into the septa, M is the amount of pheromone remaining in the septa at the start of the observation period, and $t^{1/2}$ is the half-life of the active ingredient, which in the case of *Z*8–12:OAc is 36.8 days (McDonough and Butler, 1983; McDonough et al., 1989). Calculated release rates per septum after five days for septa loaded with 0.01, 0.1, 1, 10, 100, and 1000 μg of *Z*8–12:OAc were 7.1 pg/hr, 71.4 pg/hr, 714 pg/hr, 7.1 ng/hr, 71.4 ng/hr, and 714.0 ng/hr, respectively. These figures are approximately three times higher than emission rates from rubber septa obtained by Baker et al. (1980) by direct measurement of adsorption on glass surfaces.

Wind Tunnel. The wind tunnel used in these experiments has been described previously (Sanders, 1982). It is constructed of plate glass and is 90 cm × 90 cm in cross section and 2 m long. Wind speed was about 50 cm/sec and the light intensity about 35 lux. For these experiments, as with previous experiments on spruce budworm (Sanders, 1996), a sheet of plate glass was mounted horizontally in the tunnel as an artificial floor, separating the tunnel vertically into two equal halves. A wire frame was placed in the top half with 16 locations marked on it for mounting the rubber septa. These were positioned in four rows, A, B, C and D, which were 100, 124, 148, and 172 cm from the downwind end of the tunnel, respectively. Rows A and C had six septa in a 2 × 3 grid, the top three septa were 10 cm from the roof, the bottom 10 cm up from the floor, with two septa on the centerline of the tunnel and the others 12.5 cm in

from the sides. Rows B and D had two septa, each positioned at mid-height of the half tunnel, 30 cm in from opposites sides. Each septum was backed by a square of paper, 2.5 × 2.5 cm, to create a turbulent plume.

Methods. At 11:00 hr on the day of each experiment, rubber septa loaded with the appropriate concentration of the synthetic pheromone were pinned to each of the 16 locations on the wire frame in the upper half of the tunnel. At the same time, two 2-day-old virgin females were placed in small screen cages, 3 cm in diameter and 2.5 cm long, which were positioned on a stand in the upper half of the tunnel within the array of rubber septa, 30 cm from the upwind screen, halfway between the artificial floor and the roof, and 30 cm in from one side of the tunnel. Also at 11:00 hr on the day of each experiment, ca. 30 2-day-old males were placed individually in small screen cages, half of which were placed on a rack in the lower half of the tunnel, the other half on a rack in the upper half of the tunnel. The males in the upper half of the tunnel were placed on the opposite side of the tunnel to the target females. This ensured that they were not exposed to the plume from the target females, although they were continually exposed to synthetic pheromone from the septa.

Starting at 14:00 hr, the cages containing the males were moved one at a time directly downwind of the target females, 30 cm in from the end of the tunnel. The lid was removed from the cage and the following behaviors of each male were recorded and timed: (1) wing-fanning, (2) taking flight, (3) locking on to the female plume and passing a point 50 cm from the downwind end of the tunnel, (4) proceeding upwind past a point 100 cm from the downwind end of the tunnel (row A of the septa), and (5) landing on a septum or the cages housing the female moths. Each male was allowed 1 min to respond, and each assay was terminated if the male landed on an inside surface of the tunnel and remained inactive for >10 sec.

Analyses. Comparisons of numbers of males responding were carried out by the G test for homogeneity (Sokal and Rohlf, 1981). Comparisons among response times were carried out by first transforming the data to logarithms, which normalized the distributions, and then subjecting the transformed data to multivariate analyses, using the Minitab GLM procedure (Minitab Inc., State College, Pennsylvania). Standard deviations were calculated from the untransformed data.

RESULTS

Responses to Single Lures and Females with No Background of Synthetic Pheromone

Maximum levels of response were evoked by septa loaded with 1 or 10 μg of the synthetic pheromone, and these were not significantly different from

TABLE 1. PERCENTAGES OF MALE ORIENTAL FRUIT MOTHS RESPONDING IN WIND TUNNEL TO TWO CAGED, VIRGIN FEMALE MOTHS OR TO SINGLE RUBBER SEPTA LOADED WITH DIFFERENT AMOUNTS OF SYNTHETIC PHEROMONE[a]

Lure	N	Wing-fanning (%)	Response (%) after exposure to lure		
			Quiet	Flying to side	Landing on lure
Two caged females	38	97.4a	5.3c	0b	94.7a
Septum					
0.01 μg	38	10.5c	52.6a	15.8b	31.6b
0.1 μg	37	59.5b	13.5b	5.4b	81.1a
1.0 μg	48	97.9a	4.2c	0b	95.8a
10.0 μg	54	92.6a	11.1c	0b	88.9a
100 μg	45	95.6a	0c	66.7a	33.3b
1000 μg	22	95.5a	9.1c	81.8a	9.1b

[a]Numbers in the same column followed by the same letter are not significantly different (G^2 test of homogeneity, $P = 0.05$).

response levels to the two caged virgin female moths (Table 1). At lower loadings, 0.01 and 0.1 μg, the number of moths wing-fanning in response to the septa declined significantly. Furthermore, among those males that did wing-fan in response to the two lower loadings, the latency period until the start of wing-fanning was longer than in response to either the caged females or to the higher loadings of synthetic pheromone; there was also a tendency for the males to fly upwind more slowly in the presence of the lower loadings (Table 2). Above the optimum loadings, significantly more males showed arrestment of upwind flight, which resulted in the moths veering off to the inside surfaces of the wind tunnel without reaching the pheromone sources (Table 1). Again there was a tendency for the males to fly more slowly as the loadings of synthetic pheromone increased above the optimum (Table 2).

Responses in the Presence of Backgrounds of Synthetic Pheromone

Wing-Fanning. Most of the males that had been kept in the lower half of the tunnel in pheromone-free air (naive males) wing-fanned immediately when moved to the upper half of the tunnel, downwind of the target females, regardless of the background concentration (Tables 3 and 4, Figure 1). Among the experienced males that had been preexposed to synthetic pheromone in the top half of the tunnel, most responded immediately at the lower concentrations (10 μg and lower), but at the higher concentrations significantly fewer wing-fanned,

TABLE 2. TIMED RESPONSES ($\bar{x} \pm$ SD) OF MALE ORIENTAL FRUIT MOTHS IN WIND TUNNEL TO TWO CAGED, VIRGIN FEMALE MOTHS OR TO SINGLE RUBBER SEPTA LOADED WITH VARIOUS AMOUNTS OF SYNTHETIC PHEROMONE[a]

Lure	Start of wing-fanning		Passing midpoint of tunnel		Passing 150 cm mark	
	N	Seconds	*N*	Seconds	*N*	Seconds
Two caged females	37	0.69 ± 0.87b	36	1.56 ± 2.25b	27	5.08 ± 4.34b
Septum						
0.01 μg	5	9.05 ± 19.0a	12	4.34 ± 6.09a	5	13.79 ± 1.69a
0.1 μg	27	1.00 ± 3.75b	35	3.18 ± 4.77a	15	8.98 ± 6.33ab
1.0 μg	43	0.65 ± 1.68b	46	3.27 ± 5.11a	26	8.75 ± 5.83ab
10 μg	44	0.86 ± 3.12b	49	2.24 ± 3.71ab	29	6.65 ± 3.56b
100 μg	43	0.64 ± 1.74b	43	2.47 ± 3.45ab	17	7.96 ± 2.86ab
1000 μg	21	0.60 ± 0b	16	2.99 ± 6.08ab	3	10.13 ± 2.74ab

[a]Numbers in the same column followed by the same letter are not significantly different (Tukey's procedure, $P = 0.05$).

and those that did wing-fan took significantly longer to respond (Tables 3 and 4, Figure 1).

Success in Locating Females. The numbers of males able to locate the female moths decreased as the concentration of synthetic pheromone in the septa was increased (Table 3, Figure 1), but the decreases were not significant until loadings were greater than 1 μg/septum (time-averaged atmospheric concentration 20 pg/m^3). At a loading of 1000 μg (time-averaged atmospheric concentration about 20 ng/m^3), virtually no males reached the virgin females (i.e., disruption was complete).

At the lower loadings (10 μg/septum or less) those males that did not locate the females went to septa instead (i.e., false-trail following). However, at the two highest loadings, 100 and 1000 μg/septum, the majority of the disrupted males were either inactive and did not fly, or if they flew they ceased upwind flight and eventually flew to the sides of the tunnel without reaching either septum or females. In this there were significant differences in behavior between the experienced and naive males. The majority of the naive males at both the 100- and 1000-μg loadings took flight but failed to sustain upwind flight. The same pattern occurred among the experienced males with the 100-μg loading, but at the 1000-μg loading the majority of experienced males remained inactive and did not take flight. Among both naive and experienced males the durations of flight before the males alighted on the inner surface of the tunnel were significantly shorter at the 1000-μg loading than at the 100-μg loading.

TABLE 3. PERCENTAGES OF MALE ORIENTAL FRUIT MOTHS RESPONDING IN WIND TUNNEL DOWNWIND OF TWO CAGED, VIRGIN FEMALE MOTHS SURROUNDED BY RUBBER SEPTA LOADED WITH VARIOUS AMOUNTS OF SYNTHETIC PHEROMONE[a]

Treatment and loading (μg/septum)	N	Wing-fanning	Quiet	Landing		
				On side of tunnel	On septum	On female cage
Two caged females with no disruptant	38	97.4ab	5.3cdef	0c		94.7a
Experienced males						
0.01	35	97.1abc	2.9cdef	0c	14.3ef	82.8a
0.1	120	98.3a	0.8def	2.5c	15.0ef	81.7a
1.0	134	91.8abc	5.2cdef	0c	45.5bcd	49.3bc
10	144	84.7bcd	9.0bcde	2.1c	64.6ab	24.3cde
100	122	63.9de	23.8b	58.1ab	6.6efg	11.5ef
1000	100	31.0f	59.0a	41.0b	0g	0g
Naive males						
0.01	33	78.8cd	9.1bcd	0c	21.2cde	69.7ab
0.1	113	39.8ef	0f	0.9c	21.2cde	77.9a
1.0	122	99.2a	0.8def	0c	58.2abc	41.0bcd
10	139	99.3a	0.7ef	4.3c	71.3a	23.7de
100	122	96.7ab	1.6cdef	68.9ab	19.7e	9.8efg
1000	96	96.9abc	14.6bc	82.3a	2.1fg	1.0fg

[a]Half the males were exposed in the wind tunnel to the ambient pheromone conditions for 3 hr before assays (experienced males), the other half (naive males) were kept in pheromone-free air. Numbers in the same column followed by the same letter are not significantly different (G^2 test of homogeneity, $P = 0.05$).

DISCUSSION

In order to compare the results of these experiments on Oriental fruit moths with previous work on spruce budworms (Sanders, 1996), comparable data are required on release rates of the chemicals from the rubber septa. No direct measurements have been made of release rates of spruce budworm pheromone from rubber septa. Therefore, release rates were calculated by the Butler and McDonough (1979) method. Release rates of Z8–12:OAc, the major pheromone component of the Oriental fruit moth, calculated by the Butler and McDonough (1979) method, are approximately three times higher than those obtained by glass-surface adsorption by Baker et al. (1980). Reasons for these differences are not obvious. They may be because of differences in the characteristics of the septa or because the loaded septa used by Baker et al. (1980) were aged for

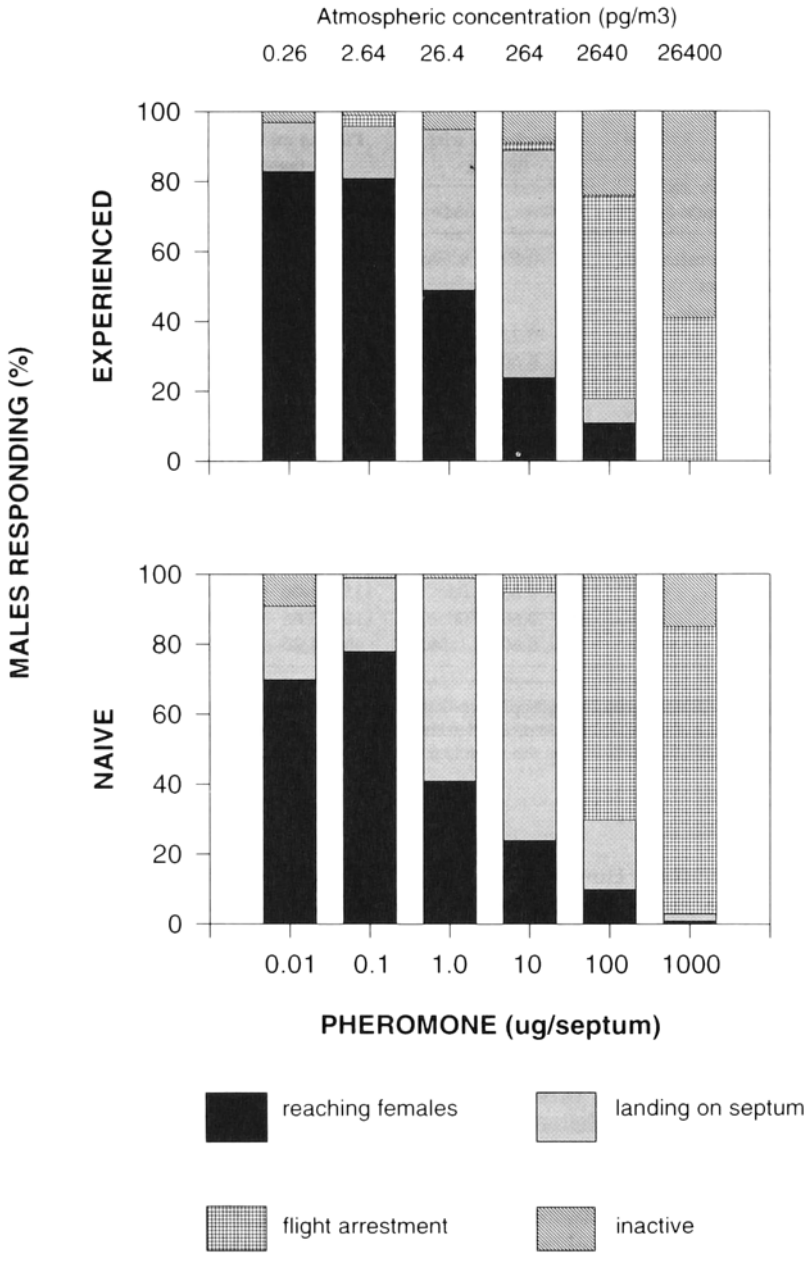
TABLE 4. TIMED RESPONSES ($\bar{x} \pm$ SD) OF MALE ORIENTAL FRUIT MOTHS EXPOSED IN WIND TUNNEL TO TWO CAGED, VIRGIN FEMALE MOTHS SURROUNDED BY RUBBER SEPTA LOADED WITH VARIOUS AMOUNTS OF SYNTHETIC PHEROMONE[a]

Treatment and loading/septum (μg)	Start of ning wing-fanning		Passing midpoint of tunnel		Landing on side of tunnel	
	N	Seconds	*N*	Seconds	*N*	Seconds
Two caged females with no disruptant	37	0.69 ± 0.87cd	36	1.56 ± 2.25d	0	
Experienced males						
0.01	33	0.72 ± 2.52bcd	34	3.52 ± 4.98bcd	0	
0.1	117	0.60 ± 0.78d	116	3.72 ± 1.62bc	0	
1.0	110	0.72 ± 3.24cd	127	3.60 ± 5.40bc	0	
10	123	1.08 ± 8.46b	129	4.20 ± 3.36b	7	
100	78	0.90 ± 3.72bc	76	6.24 ± 10.32a	70	39.8 ± 32.0a
1000	29	2.16 ± 9.24a	19	4.80 ± 10.92ab	39	31.1 ± 18.5b
Naive males						
0.01	33	0.82 ± 4.74bcd	30	3.84 ± 2.46bc	0	
0.1	113	0.60 ± 0d	113	3.60 ± 1.62bc	1	
1.0	122	0.60 ± 0.12d	120	2.70 ± 1.50cd	0	
10	139	0.60 ± 0d	135	3.06 ± 2.40bcd	6	
100	122	0.60 ± 0d	116	3.66 ± 4.92bc	84	39.7 ± 29.2a
1000	96	0.60 ± 1.74d	46	4.26 ± 10.2b	80	30.5 ± 16.2b

[a]Males were divided into two groups, one kept for 3 hr in presence of the ambient pheromone (experienced males), the other kept in pheromone-free air until exposure (naive males). Numbers in the same column followed by the same letter are not significantly different (Tukey's procedure, $P = 0.05$).

only 12 or 16 hr. However, for comparative purposes, the calculated release rates by the Butler and McDonough (1979) method will be used here.

When presented with single septa (i.e., a nondisruptive situation) in the wind tunnel, male Oriental fruit moths responded optimally to loadings of 1 or 10 μg, and the responses at these loadings were not significantly different from responses to two virgin female moths. These results conform well to previously published results with single septa. Baker et al. (1981), Baker and Haynes (1989), and Linn et al. (1988) all showed that the optimum loadings of synthetic Oriental fruit moth pheromone containing about 6% of the *E* isomer to be between 3 and 30 μg/septum for upwind flight of the male moths. Release rates from septa loaded with 1 and 30 μg, calculated by the Butler and McDonough (1979) method are between 1 and 20 ng/septum/hr, which spans the release rates of pheromone from calling female Oriental fruit moths (Baker et al., 1980;


Atmospheric concentration (pg/m3)
0.26
2.64
26.4
264
2640
26400
MALES RESPONDING (%)
EXPERIENCED
NAIVE
100
80
60
40
20
0
0.01
0.1
1.0
10
100
1000
PHEROMONE (ug/septum)
reaching females
landing on septum
flight arrestment
inactive

Lacey and Sanders, 1992). The fact that the responses at the loadings of 1 or 10 μg were not significantly different from responses to two virgin female moths supports the conclusion that the three-component pheromone blend is very close in composition to the female-released blend.

Flight arrestment to single septa became significant at a loading of 100 μg and complete arrestment occurred at 1000 μg. Baker et al. (1981) and Linn et al. (1988) found significant arrestment at the 30-μg loading, with very high levels of arrestment at 100-μg and 300-μg loadings. These loadings correspond to calculated release rates of 70 ng/septum/hr, which is only three times that of the highest release rates recorded from individual female moths (Lacey and Sanders, 1992), and 700 ng/septum/hr, respectively.

In the case of the spruce budworm, optimal responses occurred with loadings of 1 μg up to 1000 μg/septum (Sanders, 1992). Furthermore, even at the highest loading tested against male spruce budworm, 1000 μg/septum, there was no evidence of flight arrestment (Sanders, 1992). The half-life of a 14-carbon aldehyde in a rubber septum is 43 days (Heath et al., 1986) and for (*Z*)-8-dodecenyl acetate, 36.8 days (McDonough et al., 1989). The release rates will therefore be very similar, which indicates that male spruce budworms can orient upwind in much higher concentrations of pheromone than can male Oriental fruit moths. However, there is evidence that the identified pheromone of the spruce budworm is incomplete (Alford et al., 1983; Sanders, 1984), and whether the same levels of behavior would occur in the presence of a complete pheromone blend remains to be seen.

The three-component synthetic pheromone blend of the Oriental fruit moth evokes the full suite of behavioral responses (Baker and Cardé, 1979a; Baker et al., 1981; Linn et al., 1986, 1987), which suggests that it is close to the natural pheromone. However, as the wind-tunnel observations of Witzgall (1996) have shown, there is the possibility that additional minor components remain unidentified. The ultimate question is whether or not male moths can locate calling females, not just rubber septa. Therefore, in both the previous experiments on the spruce budworm and those described here on the Oriental fruit moth, the ability of male moths to locate virgin female moths was used to assess

FIG. 1. Responses of male Oriental fruit moths in a wind tunnel to two female moths surrounded by rubber septa loaded with synthetic pheromone. The upper graph is of males kept in the upper half of the wind tunnel where they were exposed to the synthetic pheromone continually for 3 hr before being moved downwind of the two caged females (experienced males), the lower graph is of males kept in the lower half of the tunnel in pheromone-free air before being moved to the upper half, downwind of the two caged females (naive males).

disruption. As in the previous work on spruce budworms, effects on male Oriental fruit moths were concentration dependent. At release rates below those of calling female moths (the 0.01 and 0.1 μg loadings), there were no reductions in the numbers of males orienting to the females. At release rates approximating those of the calling females (the 1.0- and 10-μg loadings), males were still able to orient (i.e., there was no evidence of habituation), but as many or more males went to the septa as to the females, which confirms that at these loadings the septa were competitive with the females. When the females were surrounded by septa loaded with 100 or 1000 μg, disruption was almost complete and, as would be expected from the results with the single septa, most males were either completely inactive or their upwind flight was arrested, both of which indicate habituation.

Of particular interest is the fact that disruption measured as the reduction in numbers of male Oriental fruit moths reaching the females was not significantly different between the experienced and naive males for any of the loadings of synthetic pheromone. However, significantly more experienced males remained completely inactive at the higher loadings than did naive males. This indicates that exposure of male Oriental fruit moths to ambient pheromone for a few hours increases the level of habituation as was shown by Figueredo and Baker (1992), and as was also found in similar experiments with the spruce budworm (Sanders, 1996).

Field trials have indicated that the spruce budworm is not as susceptible to mating disruption as the Oriental fruit moth (Sanders, 1979; Sanders and Seabrook, 1982; Vickers, 1990; Rice and Kirsch, 1990; Rothschild and Vickers, 1991). The major difference in the behavior of the two species in these wind-tunnel experiments is the high levels of habituation (inactivation and flight arrestment) among the Oriental fruit moth males in the presence of septa loaded with 100 and 1000 μg compared to the spruce budworm (Figure 2), where at the same loadings few male spruce budworms showed evidence of habituation.

It is now well established that successful upwind orientation by male moths to pheromone sources depends upon fluctuations in pheromone concentration caused by air turbulence (e.g., Baker and Haynes, 1989; Vickers and Baker, 1994; Mafra-Neto and Cardé, 1995). Moreover, pulsing signals are more effective than continuous signals in causing habituation (Bartell and Lawrence, 1976; Kuenen and Baker, 1981). It is also probable that in any operational mating disruption program there will be fluctuations in concentrations of the synthetic pheromone that allow male moths to successfully orient and fly upwind (false-trail following). In recognition of this, turbulence was deliberately created in these wind-tunnel experiments. Unfortunately, the measurement of instantaneous pheromone concentrations in turbulent air is not simple (e.g., Rumbo et al., 1995), and for designing mating disruption programs it is more practical to

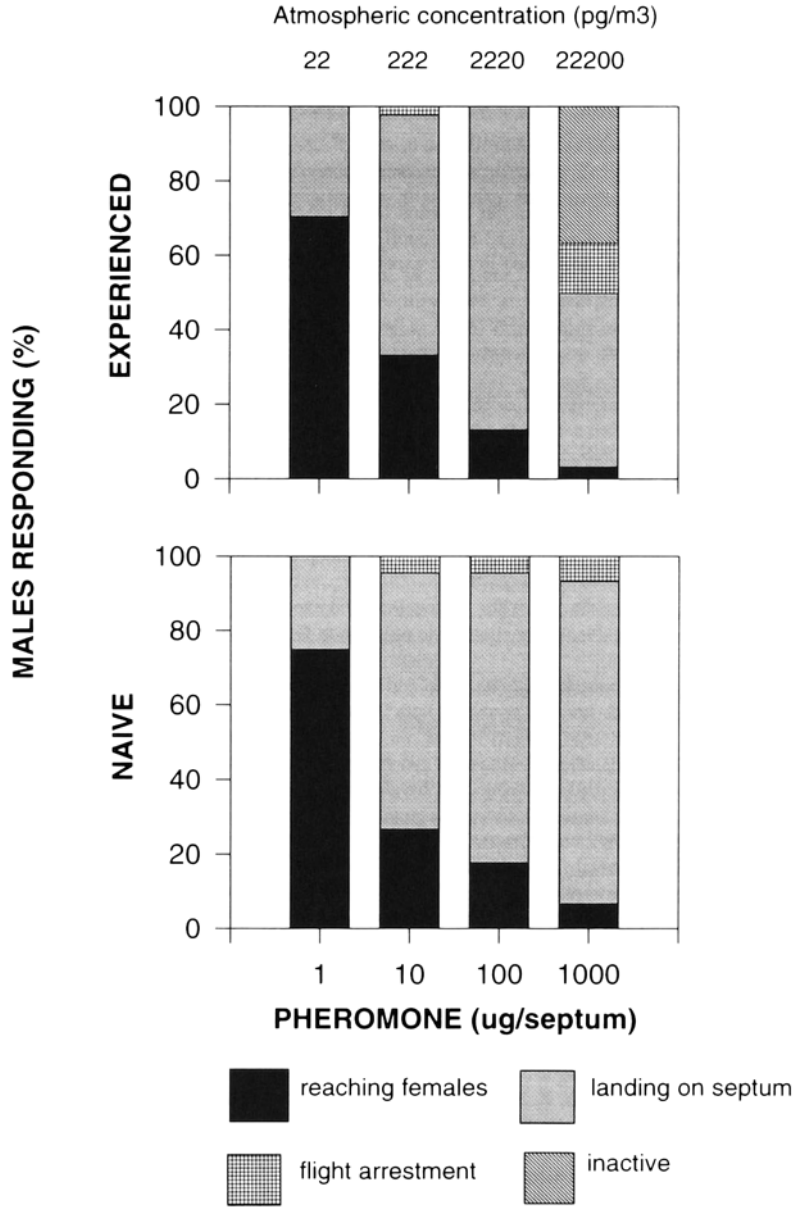


FIG. 2. Responses of male spruce budworm moths in a wind tunnel under the same conditions as those for oriental fruit moth in Figure 1. Modified from Sanders (1996).

define time-averaged concentrations with the assumption that turbulence will create the necessary fluctuating signals. The results with the spruce budworm (Sanders, 1996) and also with the Oriental fruit moth reported here suggest that for effective disruption, time-averaged atmospheric concentrations of synthetic pheromone must exceed 20 ng/m^3.

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THE ROLE OF PRIMARY AND SECONDARY METABOLITES IN CHRYSANTHEMUM RESISTANCE TO *Frankliniella occidentalis*

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Abstract—The presence of deleterious secondary metabolites to western flower thrips (WFT) (*Frankliniella occidentalis* Pergande) in several chrysanthemum (*Dendranthema grandiflora* Tzvelev) cultivars was tested. WFT performance on leaf sap mixed with artificial diet was compared to WFT performance on a control of artificial diet mixed with water. Leaf sap of six of 10 chrysanthemum cultivars tested had a significant negative effect on WFT performance and therefore contained deleterious secondary metabolites. Leaf sap of a resistant and a susceptible chrysanthemum cultivar were fractionated by gel to study the role of secondary metabolites in greater detail. None of the 20 individual fractions obtained had a negative effect on WFT performance, whereas after mixing of these fractions activity was found again. Moreover, WFT performance on the fraction mixture of the resistant cultivar was significantly lower than on the susceptible fraction mixture. The results suggest that WFT resistance in the cultivars studied was caused by several secondary metabolites that had an additional or synergistic negative effect on WFT performance. The role of a deficiency of primary metabolites in WFT resistance of chrysanthemum was also investigated. WFT performance on leaf sap with and without extra primary metabolites (artificial diet) was compared. The variance in WFT performance was significantly smaller when extra primary metabolites were added; WFT performance on leaf sap with and without extra primary metabolites was not correlated. The results suggest that resistance was partly caused by a deficiency of primary metabolites.

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Key Words—Chrysanthemum, *Dendranthema grandiflora*, western flower thrips, *Frankliniella occidentalis*, host plant resistance, chemical defense, artificial diet, synergism, primary metabolites, secondary metabolites.

INTRODUCTION

In chrysanthemum (*Dendranthema grandiflora* Tzvelev) 76% of the variation in resistance among cultivars to western flower thrips (WFT) (*Frankliniella occidentalis* Pergande) could be explained by the variation in chemical composition of the leaves (De Jager et al., 1995b). This was concluded from a highly significant positive correlation found between WFT performance on intact leaves and on leaf sap of 10 different chrysanthemum cultivars. The compounds responsible for WFT resistance are unknown.

The majority of the studies that have been reported on the chemical mechanisms of insect resistance are concerned with single compounds or single classes of compounds that deter insect feeding (Berenbaum and Zangerl, 1992; Rosenthal and Berenbaum, 1991; Kennedy and Barbour, 1992). Two major problems are involved with this single compound approach. First, because many different chemical compounds are known to have a deleterious effect on insect performance, it is difficult to select only one particular compound to study the mechanism of insect resistance (Berenbaum and Zangerl, 1992). Second, some studies proved additional or even synergistic effects between different compounds that can easily be overlooked when single compounds are studied (Berenbaum and Neal, 1985; Stadler and Buser, 1984; Hay et al., 1994). We prefer to start with total plant extracts that are fractionated until the chemicals responsible for insect resistance are identified (Lewis and Van Emden, 1986).

Primary metabolites are generally beneficial and may also be involved in insect resistance if they occur in limiting concentrations (Bernays and Chapman, 1994; Slansky, 1992). Therefore, along with secondary metabolites, the role of primary metabolites also should be studied.

In this study, the mechanism of chemical resistance to WFT in chrysanthemum was investigated. First, the role of deleterious secondary metabolites was studied by measuring WFT performance on leaf sap of several chrysanthemum cultivars mixed with artificial diet. In more detailed analyses, leaf sap of a resistant and a susceptible cultivar was fractionated by means of gel filtration and WFT performance on the different fractions was measured. Relative larval growth and survival after three days were used as measures of WFT performance. These parameters had proved to be quick and reliable measures (De Jager et al., 1995b). To study the role of primary metabolites in WFT resistance, performance of WFT larvae on leaf sap with and without extra primary metabolites (artificial diet) was compared.

METHODS AND MATERIALS

Plant and Insect Source. Ten chrysanthemum cultivars covering a large range in resistance were selected for the experiments (De Jager et al., 1995a). The cultivars, referred to as cv. 1, 2, 3, 5, 6, 9, 11, 14, 15, and 31, were coded by the breeding companies Fides Research and Breeding B.V. (De Lier, The Netherlands) and Chrysanthemum Breeder Association N.V. (Ter Aar, The Netherlands).

Commercially produced chrysanthemum cuttings were dipped in Savona (a nonpersistent mixture of natural fatty acids; Koppert, Berkel en Rodenrijs, The Netherlands) to kill pest insects. Next, the cuttings were dipped in Rapid root 1 (rooting powder with 0.1% 3-indolylbutyric acid; Koninklijke Brinkman, Maasland, The Netherlands) to stimulate root growth and planted in small compost blocks of 64 cm^3. Desiccation and burning of the cuttings were prevented by covering the cuttings with plastic and a layer of filter paper. This cover was removed after two weeks. The rooted cuttings were planted in 9-cm-diameter pots with a 3:1 compost-vermiculite mixture. Plants were placed at random in the growth room. From then on, the plants received 75 ml of nutrient solution (15:20:25 NPK; EC = 2 mS/cm) once a week. The plants were watered when necessary to keep the compost humid. The growth room with mercury lamps (27 W/m^2) was programmed for a 16L:8D photoperiod, 20°C, and a relative humidity of 70%. After four weeks, light conditions were changed from long day length to short day length (8L:16D) as is the practice in chrysanthemum breeding to induce flowering.

The laboratory colony of WFT used for the experiments was reared for several months on the flowers of a susceptible cultivar (cv. 8), grown under commercial greenhouse conditions. For verification of the thrips species, a sample of the laboratory colony was regularly sent to G. Vierbergen, a thrips specialist of the Plant Protection Service (Wageningen, The Netherlands). The WFT were reared in a growth room with fluorescent lamps (58 W, white 33, Splendor), a 12L:12D photoperiod, 20°C continuously, and a relative humidity of 65%. For all experiments 0-to 1-day-old larvae were used. To obtain these larvae, fresh flowers were put in the rearing cages six days before the beginning of the experiments. At the beginning of the experiment, the flowers were torn apart and the first instars were collected.

Artificial Diet. A multiorder diet as described by Singh (1983) was modified to prepare a liquid artificial diet on which WFT could feed and grow. We only used the soluble ingredients of the multiorder diet (Table 1) since that would facilitate the ingestion of the food by WFT. The diet was prepared at a concentration two times higher than described by Singh (1983). In tests it was mixed (1:1 v/v) with different extracts, which finally resulted in a concentration

TABLE 1. COMPOSITION OF ARTIFICIAL DIET FOR *F. occidentalis*

Group	Ingredient	Quantity (w/w)
1	Casein	7.0%
	Wesson's salt	2.0%
2[a]	Cholesterol	0.1%
	Linoleic acid	0.5%
3[b]	Vanderzant vitamin mix	4.0%
	Sucrose	6.0%
	Distilled water	80.4%

[a]Ingredients of group 2 were dissolved in 50 ml dichloromethane, mixed with group 1, and then dichloromethane was evaporated.
[b]Ingredients of group 3 were dissolved in H_2O, mixed with groups 1 and 2, and then the pH was adjusted to ca. 6.5 with 4.0 M KOH.

of the added soluble ingredients that was exactly the same as described by Singh (1983). To study the nutritional value of the artificial diet, WFT performance on artificial diet and other chrysanthemum diets was compared.

Secondary Metabolites. Twenty cuttings of each of the 10 selected cultivars were grown in a growth room as described in the first section. After five weeks, all leaves of 10 plants per cultivar were harvested and leaf sap was separated from the largest leaf fragments in a food processor. The leaf sap was stored for several days at −80°C. To test WFT performance on leaf sap with about the same concentration of leaf constituents as in intact leaves, the sap was concentrated twice by freeze-drying prior to mixing it (1 : 1 v/v) with artificial diet.

Leaf Sap Fractionation. One hundred cuttings of chrysanthemum cultivars 2 (resistant) and 15 (susceptible) were grown as described in the first section. After six weeks, the leaves of all plants were harvested and put in a food processor where the leaves were mashed to make leaf sap. Leaf sap could be collected from a cup at the bottom of the processor that was placed under a sieve. The leaf sap was stored for several weeks at −20°C.

Prior to fractionation a dose–response test was performed for both the resistant and the susceptible cultivar. A mixture of artificial diet and leaf sap (1 : 1 v/v), with final concentrations of 0.5, 1, and 2 times the starting concentration of leaf sap, was fed to WFT larvae in the bioassay.

Fractionation of leaf sap was performed by gel filtration. The gel filtration method was chosen because there was no knowledge of the type of chemical compound(s) causing WFT resistance in chrysanthemum. Gel filtration is a fast method to separate chemical compounds and gives a minimum chance of specific binding to the column. Moreover, it was possible to use water as eluent, which is the most common compound of leaves. Sixty grams of Sephadex LH-20 gel

(Pharmacia) was swollen in about 400 ml distilled water for one night at 4°C. The gel was poured in a column with a length of 45 cm and a diameter of 3 cm. The homogeneity and reproducibility of the packed column was confirmed with 15 ml of a solution of blue dextran (4.7 mg/ml) and NaCl (0.5 M). Water was used as eluent.

Leaf sap was centrifuged for 10 min at 18,000*g* at room temperature to remove leaf particles. Leaf sap of cv. 2 and cv. 15 was concentrated 6.9 and 7.9 times, respectively, by freeze-drying. The concentrated sap (8.7 g of cv. 2 and 7.6 g of cv. 15) was placed on top of the gel for fractionation. Water was used as eluent and 30 fractions of about 15 ml each were collected and weighed. The fractionation was performed at 4°C. Absorbance was measured at 280 nm. On the basis of the absorbance it was decided to measure the effect on larval performance of fractions 6–25 of cv. 2 and 6–23 of cv. 15. During fractionation of cv. 2, a precipitate was formed on top of the column. The leaf sap was allowed to enter the column and was eluted with about 60 ml water, after which the precipitate was removed. The effect of all fractions and the precipitate on WFT performance was tested as described in the bioassay section. One milliliter of all fractions was taken for the bioassay. The remains of the fractions were freeze-dried in order to determine the dry weight to fresh weight (DW/FW) ratio. With these ratios the concentration of leaf constituents tested was estimated to be about 0.7 and 0.8 times the concentration in intact leaves of cv. 2 and cv. 15, respectively.

The fractionation by gel filtration was repeated with minor changes. In the second experiment, 5 ml of 13.4 and 13.7 times concentrated leaf sap of cv. 2 and 15, respectively, was placed on top of the column. A precipitate was formed during fractionation of the leaf sap of both cultivars. To test whether any activity was lost during fractionation, a mixture was made with 1 ml of each of fractions 6–25, freeze-dried, and resuspended in 1 ml distilled water. The effect of this mixture of WFT performance was tested in the bioassay. Prior to the bioassay, the fractions were concentrated about twofold to obtain a higher concentration of leaf constituents compared to intact leaves. On the basis of the DW/FW ratio, the test concentrations of leaf sap finally were calculated to be 2.6 and 2.9 times the normal concentrations of leaf sap of cv. 2 and cv. 15, respectively.

Primary Metabolites. Leaf sap from the 10 chrysanthemum cultivars prepared for the secondary metabolite experiment was also used for the experiment on primary metabolites. WFT performance on leaf sap mixed with water or with extra primary metabolites (artificial diet) was tested as described in the bioassay section. By addition of the artificial diet, the concentration of primary metabolites (proteins, amino acids, and sugars) was estimated at about 5 to 8 times higher than in intact chrysanthemum leaves (Soldaat, 1991; Bernays and Chapman, 1994). To get a test concentration of all other leaf constituents that was about the same as in intact leaves, the leaf sap was concentrated twice prior to

mixing it (1 : 1 v/v) with water or artificial diet. The coefficient of variation (V) was calculated for the treatment with and without extra primary metabolites and differences were tested as described by Sokal and Rohlf (1981). In addition, a Pearson correlation coefficient was calculated between both treatments.

Bioassay. Special observation plates of clear plastic were used to study larval performance on different liquid media (De Jager et al., 1995b). A liquid medium was put in the small cups of the bottom plate and covered with maximally stretched parafilm. WFT are able to feed through parafilm (Teulon, 1992; De Jager et al., 1995b). A middle plate with six holes was placed on top of the parafilm to keep the WFT larvae separated. The middle plate was covered by a top plate.

The day before the start of the experiment, the test media were put in the observation plates and stored at 4°C. On the first day of the bioassay 30 first-instar WFT larvae were introduced on each medium in the observation plates. Next, the length of each larva was measured. After two days all surviving larvae were transferred to observation plates with fresh media because in preliminary experiments we found that the quality of the media decreased after two days. The length of surviving larvae was measured again after three days. The relative growth in three days (length on day 3 divided by length on day 0) and the percentage of surviving larvae after three days was calculated for each observation plate. Relative growth and survival per observation plate were multiplied to get a general measure of larval performance (De Jager et al., 1995b). Bioassays were performed under the same conditions as the WFT were reared.

In order to compare bioassays that were performed in different weeks, a control medium of artificial diet and distilled water was included in each bioassay and WFT performance was calculated relative to the control. Differences between the media in larval performance was tested by ANOVA followed by Duncan's multiple-range test. To test whether WFT performance was significantly lower on the fraction mixture of the resistant cultivar (cv. 2) than on the susceptible cultivar (cv. 15), an unpaired, one-tailed Student's t-test was used (Macintosh program: Statview 512+).

RESULTS

Artificial Diet. WFT performance on artificial diet and on several chrysanthemum diets was compared. Larval performance on artificial diet was higher than on chrysanthemum leaves and leaf sap (Table 2). However, WFT performed even better on chrysanthemum petals with or without pollen (Table 2). We expect that these differences in WFT performance were mostly related to the diets because all experiments were performed with the same laboratory colony of WFT under completely similar conditions (De Jager and Butôt, 1993; De Jager et al., 1995b).

TABLE 2. RELATIVE LARVAL GROWTH × SURVIVAL OF *Frankliniella occidentalis* AFTER THREE DAYS ON DIFFERENT CHRYSANTHEMUM DIETS AND ARTIFICIAL DIET

Diet	Relative growth × survival[c]	Source
Intact leaves[a]	1.11 ± 0.07 b	De Jager et al. (1995b)
Leafsap[a]	0.77 ± 0.15 a	De Jager et al. (1995b)
Petals[a]	1.60 ± 0.04 c	De Jager and Butôt (1993)
Petals + pollen[a]	1.81 ± 0.04 c	De Jager and Butôt (1993)
Artificial diet[b]	1.26 ± 0.04 b	Present paper

[a] Mean of four different cultivars (cv. 1, cv. 2, cv. 5, and cv. 6).
[b] Mean of the experiments presented in this paper.
[c] Means indicated with the same letter are not significantly different (ANOVA: $F = 23.44$, $P < 0.0001$).

Secondary Metabolites. WFT performance on leaf sap mixed with artificial diet differed significantly between cultivars and the control ($F = 2.18$, $P = 0.038$) (Figure 1). Compared to the control of artificial diet and water, the mixtures of diet and leaf sap of five resistant chrysanthemum cultivars (2, 3, 5, 9, and 11) and one moderately resistant cultivar (31) had a significant negative effect on larval performance. No effect on larval performance was found when artificial diet was mixed with the leaf sap of two highly susceptible cultivars (6 and 15) and two other moderately resistant cultivars (1 and 14).

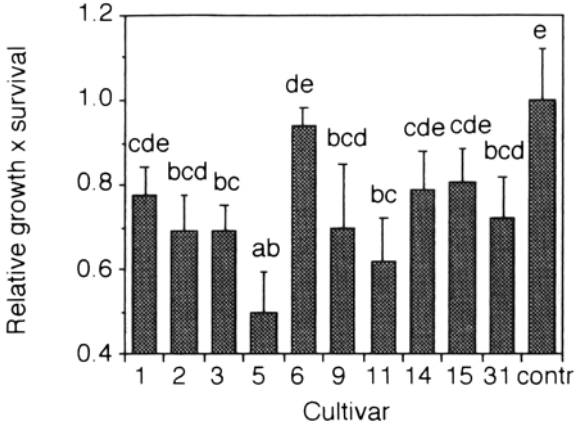

FIG. 1. Relative growth × survival (±SE) of *F. occidentalis* larvae after three days on leaf sap or water (control) mixed with artificial diet. Larval performance on 10 different chrysanthemum cultivars was compared. Means indicated with the same letter are not significantly different.

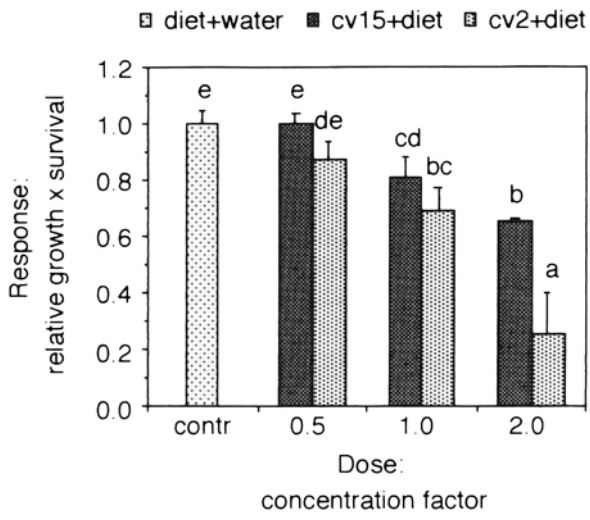

FIG. 2. Dose–response of *F. occidentalis* larvae on different dilutions of leaf sap mixed with artificial diet (1 : 1 v/v). The leaf sap of a resistant (cv. 2) and a susceptible (cv. 15) chrysanthemum cultivar was used.

Leaf Sap Fractionation. The results of the dose–response test of both the resistant and the susceptible cultivars are plotted in Figure 2. Significant differences in WFT performance were found among the different doses of leaf sap ($F = 12.4$, $P = 0.001$). Larval performance on the susceptible and the resistant cultivar only differed significantly when leaf constituents were concentrated twice compared to an intact leaf.

From the absorption patterns, it can be seen that the fractionation, both of the resistant and the susceptible cultivars, was reproducible (Figure 3). Relative larval growth × survial did not differ significantly among the individual leaf sap fractions after the first fractionation (cv. 2, $F = 1.13$, $P = 0.34$; cv. 15, $F = 0.97$, $P = 0.50$) (Figure 4). After the second fractionation, none of the individual fractions had an effect on WFT performance, although leaf constituents were tested in about a threefold higher concentration compared to the first fractionation (cv. 2, $F = 1.14$, $P = 0.33$; cv. 15, $F = 0.63$, $P = 0.88$) (Figure 4). The mixtures of fractions, however, had a significant negative effect on larval performance compared to the control (cv. 2, $F = 11.91$, $P = 0.001$; cv. 15, $F = 5.54$, $P = 0.001$). Larval performance was significantly lower on the mixture of the resistant cultivar than on the mixture of the susceptible cultivar ($t = 1.90$, $P = 0.047$).

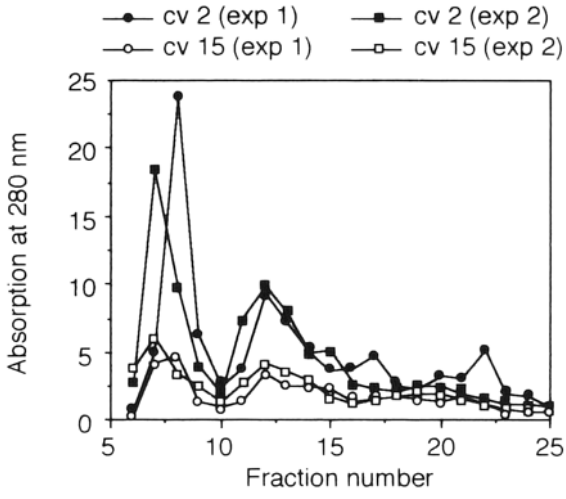

FIG. 3. Elution diagram obtained when leaf sap of a resistant (cv. 2) and a susceptible (cv. 15) chrysanthemum cultivar were fractionated. The leaf sap of both cultivars was fractionated twice (experiments 1 and 2).

Primary Metabolites. Relative growth × survival of WFT larvae differed significantly among cultivars between the treatments with and without extra primary metabolites [$F = 2.1$, $P = 0.039$ (cultivar); $F = 196.3$, $P = 0.001$ (treatment); $F = 1.1$, $P = 0.41$ (interaction)]. Addition of extra primary metabolites to chrysanthemum leaf sap had a positive effect on WFT perfor-

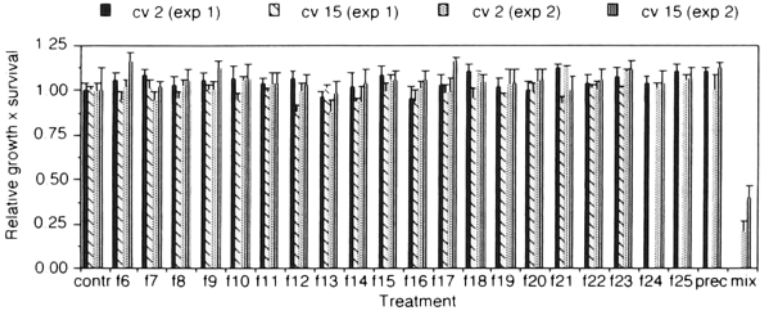

FIG. 4. Relative growth × survival (±SE) of *F. occidentalis* larvae measured on different chrysanthemum leaf sap fractions (f6–f25), a mixture of all fractions (mix), the precipitate (prec), and the control (contr). All these substrates were mixed with artificial diet. Leaf sap of a resistant (cv. 2) and a susceptible cultivar (cv. 15) were used.

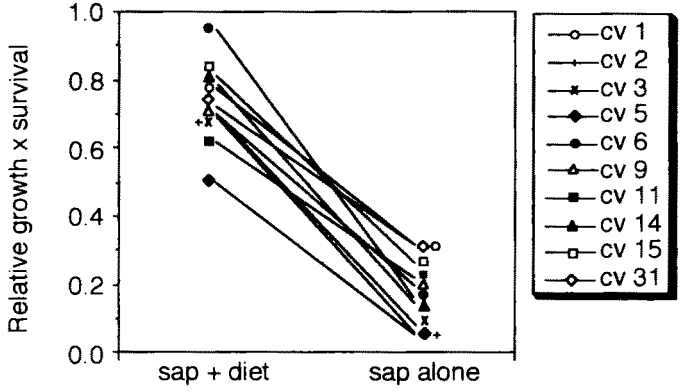

FIG. 5. Interaction between chrysanthemum cultivar and treatment (with or without primary metabolites) for relative growth × survival of *F. occidentalis* larvae.

mance. The variation in larval performance among cultivars was significantly lower ($F_s = 15.0$, $P < 0.05$) on leaf sap with extra primary metabolites ($V = 0.16$) than on leaf sap without extra primary metabolites ($V = 0.55$). The extent of the positive effect of primary metabolite addition depended on the cultivar (Figure 5). This resulted in a Pearson correlation coefficient between larval performance on both treatments that was not significant ($R = 0.16$, $P = 0.66$) (Figure 6).

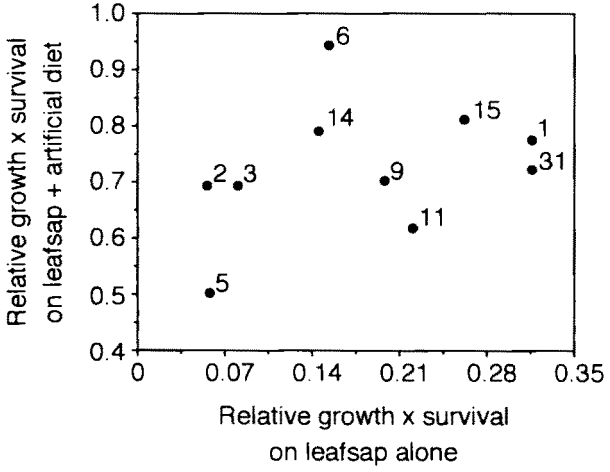

FIG. 6. Relation between WFT performance on leaf sap with and without extra primary metabolites (artificial diet). Leaf sap of 10 different chrysanthemum cultivars was used.

DISCUSSION

Leaf sap of six of 10 chrysanthemum cultivars had a significant negative effect on larval performance compared to the control. With exception of cv. 31, which is moderately resistant, intact plants of these cultivars are very resistant to WFT (De Jager et al., 1995b). Because the leaf sap was mixed with artificial diet, the negative effect could not be due to a deficiency of primary metabolites. Therefore, we concluded that deleterious compounds were present in these cultivars. For reasons of clarity, in this paper the term "secondary metabolites" is used to indicate these compounds.

None of the individual leaf sap fractions of both the resistant and the susceptible cultivars showed a negative effect on larval performance, whereas the fraction mixtures did. Moreover, larval performance on the mixture of the resistant cultivars was significantly lower than on the mixture of the susceptible cultivars. Again these effects could not be due to a deficiency of primary metabolites. Therefore, we concluded that different deleterious secondary metabolites were working additionally or synergistically in both the resistant and susceptible cultivars. The resistant cultivars appears to have more deleterious secondary metabolites than the susceptible cultivars.

The variation in resistance among cultivars was strongly reduced when extra primary metabolites in the form of an artificial diet were added. This suggests that differences between cultivars in WFT resistance were partly caused by a deficiency of primary metabolites. This agrees with the results of other studies [in rice to *Stenchaetothrips biformis* Bagnall (Thayumanavan et al., 1990); in cucumber, tomato, pepper, and lettuce to *F. occidentalis* (Mollema and Cole, 1995)]. Larval performance on leaf sap with and without extra primary metabolites was not significantly correlated. This suggests that the cultivars did not lack the same amount of primary metabolites because, if that had been the case, a positive correlation would have been found. This also suggests that the absence of primary metabolites was not related to the presence of deleterious secondary metabolites. This means that a cultivar that lacked primary metabolites independently did or did not contain deleterious secondary metabolites.

The artificial diet had a good nutritional value compared to chrysanthemum leaves and leaf sap, although feeding on petals with and without pollen resulted in a much better relative growth × survival (Table 2). The artificial diet proved to be very useful for measuring the activity of different chrysanthemum extracts to WFT larvae. The cultivars we used to perform the dose–response test differed strongly in resistance as intact plants (De Jager et al., 1995a). However, when leaf sap of both cultivars was mixed with artificial diet, significant differences in WFT performance were found only when leaf constituents at higher concentrations than in intact leaves were offered to the WFT. The activity of different chrysanthemum extracts mixed with artificial diet could best be measured in

about a twofold concentrated form compared to intact leaves. It must be kept in mind, however, that plant chemicals can have a different effect on insects when their test concentration is very different from the concentration in the plant (Lewis and Van Emden, 1986; Bernays and Chapman, 1994).

WFT resistance in chrysanthemum appears to be of a complex nature. The presence of different deleterious secondary metabolites and the absence of beneficial primary metabolites seemed to be involved. This agrees with observations that in chrysanthemum different genetic markers each explained only a low percentage of the variation in WFT resistance in a segregating F_1 family (F. Wolff, personal communication).

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SCARAB BEETLE *Anomala albopilosa albopilosa* UTILIZES A MORE COMPLEX SEX PHEROMONE SYSTEM THAN A SIMILAR SPECIES *A. cuprea*

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Abstract—Four compounds have been identified as sex pheromone constituents of the scarab beetle *Anomala albopilosa albopilosa*, namely (*R*, *Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one (buibuilactone), 2-(*E*)-nonenol, 2-(*E*)-nonenal, and methyl benzoate (in the ratio 10:3:3:1). The diel rhythm of pheromone release in *A. a. albopilosa* showed a peak at the beginning of the scotophase, which is also a peak of mating activity. On the other hand, the similar species *A. cuprea* utilizes a two-constituent sex pheromone, having a common major component, but the pheromone is released both during scoto- and photophase. Temporal difference in mating activity and pheromone release along with chemical diversity seem to form the basis for maintaining species specificity in pheromonal communication between *A. a. albopilosa* and *A. cuprea*.

Key Words—Coleoptera, Scarabaeidae, GC-EAD, methyl benzoate, 2-(*E*)-nonenal, 2-(*E*)-nonenol, (*R*, *Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one.

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INTRODUCTION

Despite the fact that some scarab beetles (Coleoptera: Scarabaeidae) in the subfamily Rutelinae utilize closely related or even the same sex pheromone compounds, temporal and/or geographical isolation plays an important role in isolating the sexual communication channel among these species. *Anomala cuprea* and *A. albopilosa sakishimana*, for example, use a mixture of two lactone compounds, buibuilactone and japonilure, but cross-attraction does not take place because the two species are geographically isolated. The former occurs on the mainland of the Japanese archipelago whereas the latter is restricted to Miyako Island in the south of Japan (Leal et al., 1994b). On the other hand, *A. octiescostata*, also occurring on the mainland, utilizes the same blend of lactones as *A. cuprea*, but they are seasonally isolated. The flight season of *A. octiescostata* is in early spring whereas that of *A. cuprea* occurs in summer (Leal et al., 1994a).

This scenario prompted us to investigate the pheromonal communication of *A. albopilosa albopilosa* Hope ("aodogane" in Japanese), a similar species that shares the same habitat and a common flight season with *A. cuprea*.

METHODS AND MATERIALS

Insects. Beetles were first collected in light traps inside Chiba Prefectural Agricultural Experiment Station. In order to start a laboratory colony, eggs laid by the field-captured females were collected daily and transferred to wet sand in ice cream cups, which were kept at 25°C. After hatching, grubs were transferred individually to ice cream cups filled with moist (or damp) leaf mold. Adults were fed on an artificial diet for wild silk moth (Nihon Chlorella Co.) at 25°C, 70% relative humidity, and 16L:8D photoperiod.

Collection of Insect Volatiles. Airborne volatiles of *A. a. albopilosa* adults, supplied only with syrup on pieces of cotton (or grape leaves), were collected on an all-glass aeration apparatus. This consisted of a 1-liter reaction flask and a two-necked flaskhead. A glass collector (1 cm ID) was packed to a depth of 1.5 cm with Super Q (Alltech, Deerfield, Illinois), which was held in place with glass wool. The collector was attached to the flaskhead with a ground glass reduction joint. The incoming air passed through similar glasss tubes packed with activated charcoal (1.2 cm ID, 30 cm) and Tenax TA (Alltech) (1 × 1 cm) and was bubbled into distilled water before entering the collection system. The reaction flask was placed inside an incubator maintained at 25°C and a 16L:8D photoperiod. The trapped materials were extracted with hexane, and the solutions were concentrated to 0.1 beetle-equivalents. In order to investigate the diel rhythm of sex pheromone release in *A. a. albopilosa*, aeration was carried out for 24 hr, with exchange of the trap columns every 2 hr.

Isolation of Pheromone. Volatiles collected from the headspace of virgin female beetles were subjected to flash column chromatography, on silica gel (Wako C-200) by successive elution with hexane–ether mixtures in the following order: 100:0, 95:5, 90:10, 80:20, 50:50, 0:100.

Indoor Bioassay. The attractancy of the synthetic sex pheromone blend was tested in a flight tunnel (4 × 1.5 m) operated at an airflow of 40 cm/sec, 0.9 lx, 78% relative humidity, and 26°C. Five experiments were conducted during the first 2 hr of the scotophase utilizing only males ($N = 5$) that were restless (the so-called "active males") at the time of the bioassays. Pieces (2 × 2 cm) of filter paper containing the synthetic blend or solvent only (control) were set in the upwind end of the tunnel at a height of 75 cm and 2.6 m away from the release point for the beetles. Males were placed individually inside the arena at a height of ca. 75 cm in a wire mesh platform and their behavior was observed for 20 min. After each assay, the test males were removed and clean air was passed through the wind tunnel at high speed for 10 min.

Analytical Procedures. Gas chromatography (GC) was carried out on a Hewlett-Packard 5890 II Plus instrument equipped with a split/splitless injector, electronic pressure control, a flame-ionization detector, and an HP 3365 Series II Chemstation. High-resolution GC analyses were performed with polar and nonpolar capillary columns: HP-Innowax and HP-5MS (30 m × 0.25 mm × 0.25 μm; Hewlett-Packard), respectively. These columns were operated at the following conditions: (1) 50°C for 1 min, increased to 180°C at a rate of 4°C/min, held at this temperature for 1 min, increased to 230°C at a rate of 10°C/min, and finally held at this temperature for 10 min; (2) 50°C for 1 min, increased to 120°C at a rate of 5°C/min, held at this temperature for 1 min, increased to 250°C at a rate of 10°C/min, and finally held at this temperature for 10 min; and (3) 70°C for 1 min, increased to 230°C at a rate of 10°C/min, and held at this temperature for 20 min. Chiral resolution was achieved on a γ-cyclodextrin-based capillary column, Chiraldex GTA (20 m × 0.25 mm × 0.25 μm; Astec, Whippany, New Jersey) operated at 150°C and 2.5 kg/cm^2 (flow rate 119 cm/sec; linear velocity 9.42 ml/min). Gas chromatography–mass spectrometry (GC-MS) was carried out either with an HP 5890 II Plus gas chromatograph linked to a mass selective detector MSD 5972 (Hewlett-Packard) or on a Hewlett-Packard gas chromatograph electron-ionization detector GCD Series. Chromatographic resolution was achieved on HP-5MS and HP-Innowax columns operated under the same conditions for GC.

RESULTS AND DISCUSSION

Analysis of airborne volatiles from male and female beetles fed only on syrup showed the occurrence of four female-specific compounds (Figure 1). The same chemical differences were observed when the beetles were fed on grape

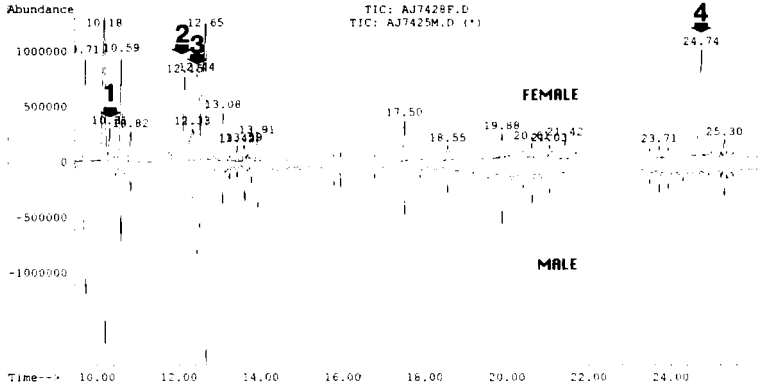

FIG. 1. Reconstructed total ion monitor chromatograms obtained from airborne volatiles of *A. a. albopilosa* virgin females (top) and unmated males (bottom).

leaves during the aeration, but the profiles were highly contaminated with compounds from the foodstuff. Utilizing a male antenna as the sensing element, GC-EAD showed that these four peaks were also EAD-active.

2,4-Hexadienoic acid, found in the extracts from both males and females, gave a distorted (leading edge) peak that interfered with detection of peaks 2 and 3 (Figure 1). This problem was overcome by collecting the airborne volatiles from females in a shorter time span (2 hr) during the time the insects were restless inside the aeration apparatus (first 2 hr after the lights went off). This gave a very clean GC-MS profile that corroborated the occurrence of only four female-specific compounds (Figure 2). Comparison of the retention times of the peaks in the male and female GC profiles may be misleading. Peak 3, for example, co-eluted with epoxilinalool (pyranoid type linalool oxide), which is produced by both male and female beetles (Figure 1). Mass spectral analyses along with the short time collection of volatiles overcame this problem and showed that in fact peak **3** is a female-specific compound.

Based on mass spectrometric data (Figure 3) and retention times on polar and nonpolar columns, the four female-specific compounds were identified as (**1**) methyl benzoate, (**2**) 2-(*E*)-nonenal, (**3**) 2-(*E*)-nonenol and (**4**) 5-(oct-1-enyl)oxacyclopentan-2-one [(*R*-buibuilactone]. We have identified the pheromone glands of scarab beetles in the subfamily Rutelinae, and the four constituents of the sex pheromone system for *A. a. albopilosa* were obtained also by direct extraction of these sex pheromone glands (Tada and Leal, unpublished data).

Two of these compounds have been previously identified as sex pheromone constituents of other scarab species. 2-(*E*)-Nonenol is a sex pheromone of *A.*

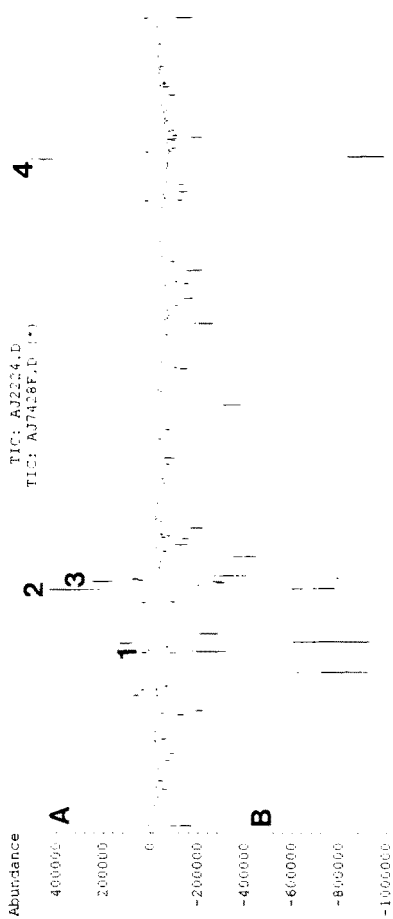

FIG. 2. Reconstructed total ion monitor profiles of the extracts of *A. a. albopilosa* virgin females collected only during hours 2 and 4 at the beginning of the scotophase (A) and for 24 hr (B).

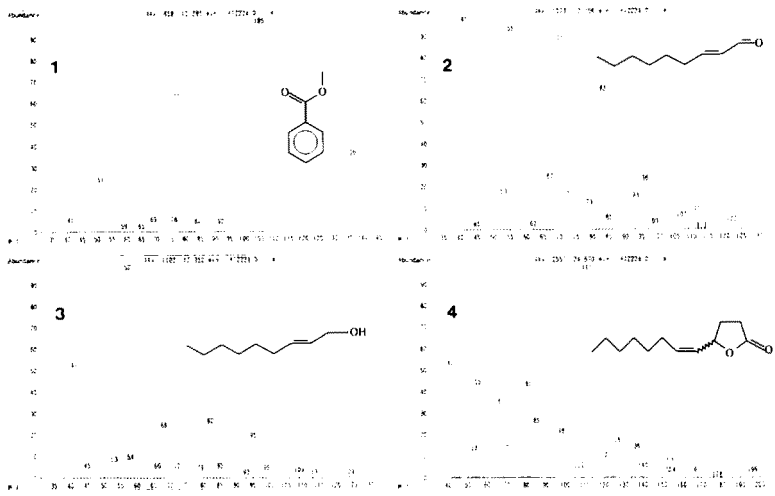

FIG. 3. Mass spectra of the four female-specific compounds identified as the sex pheromone constituents of *A. a. albopilosa*.

schonfeldti (Leal et al., 1992) and a minor constituent of the sex pheromone system of *A. daimiana* (Leal et al., 1993a). Buibuilactone, on the other hand, has been identified as a major constituent of the sex pheromone system of *A. cuprea* (Leal, 1991), *A. octiescostata* (Leal et al., 1994a), and *A. albopilosa sakishimana* (Leal et al., 1994b) as well as a minor component for *A. daimiana* (Leal et al., 1993a). In all these pheromonal systems, the natural buibuilactone had the *R* configuration. Chiral analysis of the natural product isolated by flash chromatography showed that buibuilactone from *A. a. albopilosa* also had the same stereochemistry (Figure 4). The double-bond configuration was assigned by comparison of retention times of the natural product with those of synthetic (*R*, *Z*)- and (*R*, *E*)-buibuilactone. On the basis of all data, peak 4 was fully identified as (*R*, *Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one.

The sex pheromone system of *A. a. albopilosa* is, thus, a multicomponent system. Typically, the ratio of the four constituents was 10:3:3:1 for (*R*)-buibuilactone, 2-(*E*)-nonenal, 2-(*E*)-nonenol, and methyl benzoate. The sex pheromones of rutelines are likely fatty acid derivatives (Leal, 1996), but methyl benzoate is an exception.

The behavioral response of males to a synthetic blend of the sex pheromone was observed in a flight tunnel (Table 1). Males transferred to the platform inside the flight tunnel exhibited a sequence of behavior in response to the synthetic sample. They extended their antennae with open lamellae, oriented

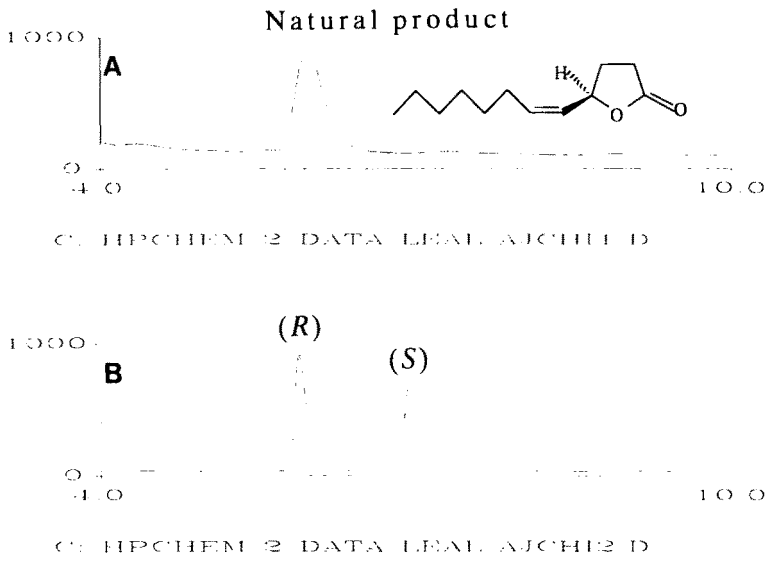

FIG. 4. Enantiomeric resolution of buibuilactone on a chiral column, Chiraldex GTA. (A) Natural product (splitless injection); (B) racemic mixture (split injection). The broader peak of the natural lactone than that of the synthetic counterpart is due to the effect of a higher solvent volume loaded on the column with the diluted natural sample.

with upwind flight, and landed on the pheromone source. After landing on the pheromone-impregnated filter paper, males searched the source with their heads very close to the source while performing abdominal movements typical of precopulatory behavior (attempting to copulate with the pheromone source). Some of the males that left the pheromone source (including those that fell down

TABLE 1. RESPONSE (%) OF MALE *A. a. albopilosa* TO SYNTHETIC SEX PHEROMONE IN A WIND TUNNEL

	Control	Pheromone[a]
Took flight	20	100
Touched source	0	96
Left source and returned	0	64
Searched on source and/or tried to copulate	0	60

[a](*R*)-buibuilactone + 2-(*E*)-nonenol + 2-(*E*)-nonenal + methyl benzoate (10:3:3:1, 1 μg = 2 FE).

during searching on the source) returned to the filter paper and started searching behavior again.

It took 3–10 min for males to reach the pheromone source after they were placed inside the arena. After locking onto the plume and proceeding in upwind flight, males were somehow attracted to the light, especially those that had left the source and were in a second flight towards the source.

Behavioral responses in a wind tunnel could not be quantified with the Japanese beetle (M. G. Klein, personal communication) and some other scarab species (Leal, unpublished data), but it has been successfully measured with other scarab species (Domek et al., 1990; Leal, 1991; Leal et al., 1992, 1993a, 1994a; Zhang et al., 1994). Nevertheless, in the present case, it has been very difficult to quantify differences in male responses to different ratios of pheromone blends not only in a wind tunnel, but also in field tests (Leal et al., 1994a). Development of more accurate bioassay(s) is needed to determine the optimum ratio of the four constituents of *A. a. albopilosa* sex pheromone system.

Although the differences in the pheromone chemistry of *A. cuprea* and *A. a. albopilosa* may play an important role in providing these two similar species with species-specific chemical signals, temporal isolation also seems to play an important role in providing isolated sexual communication channels for these two species. Female *A. cuprea* release sex pheromone both in scoto- and photophase in nearly equal amounts (Leal et al., 1993b), whereas *A. a. albopilosa* has a diel rhythm of pheromone release with a peak in the 2 hr at the beginning of the scotophase (Figure 5), although small amounts of the pheromone were also detected during the photophase. The peak of pheromone release coincided with the time females were restless (active) inside the aeration apparatus and were copulating in the rearing room. The pattern of pheromone release for the other three compounds was similar, but only the titer of the major constituent was measured.

Species specificity of the communication channel in some species is attained by temporal differences in mating activity. Flight and mating of the southern masked chafer, *Cyclocephala lurida*, begins at dusk and terminates about 2 hr later, while the peak activity of the northern masked chafer, *C. borealis*, occurs between midnight and 4 AM. Females of the two species produce a sex pheromone that is attractive to males of either species (Potter, 1980). GC-EAD experiments (unpublished data, in collaboration with Prof. Jerrold Meinwald) showed that *C. borealis* male antennae responded also to the sex pheromone released by *C. lurida* and provided further evidence that the two sympatric species share at least one sex pheromone component (Leal, unpublished data). The rhythm of pheromone release suggested that mating activity in *A. a. albopilosa* reaches a peak at the beginning of the scotophase, whereas *A. cuprea* seems to have a broader mating activity.

In conclusion, populations of *A. cuprea* and *A. a. albopilosa* utilize dif-

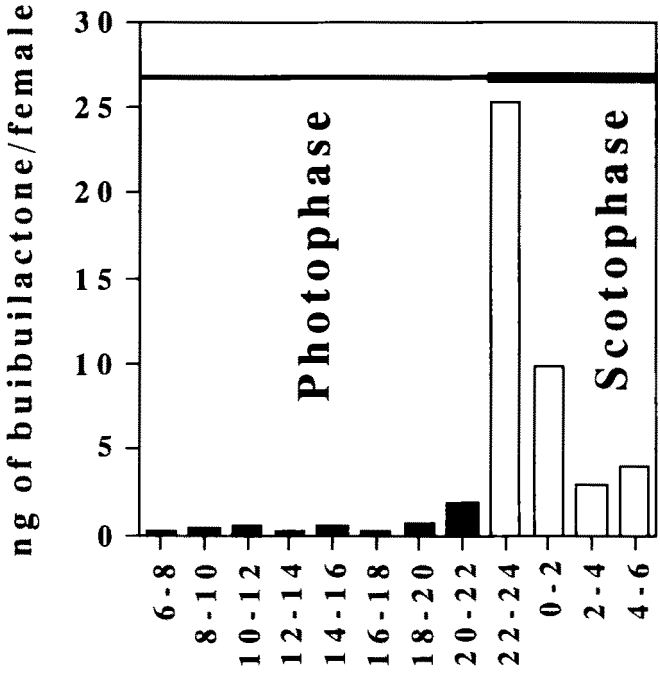

FIG. 5. Diel rhythm of *A. a. albopilosa* activity as demonstrated by the release of the major sex pheromone constituent. The profile of the other three constituents of the sex pheromone system showed a similar pattern.

ferent sex pheromone systems, with the latter having a more complex four-constituent blend. The two species have only one common constituent. This chemical diversity, along with the temporal difference in mating activity, seems to form the basis for maintaining species specificity in pheromonal communication between *A. a. albopilosa* and *A. cuprea*.

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FOOD DEPRIVATION AFFECTS PREFERENCE OF SHEEP FOR FOODS VARYING IN NUTRIENTS AND A TOXIN

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Abstract—We investigated how food deprivation affected preference of lambs for foods that varied in concentrations of nutrients and a toxin. Thirty lambs (10 lambs/treatment) were fed different amounts of alfalfa pellets (high in protein, marginal in energy for growth) as a basal ration (20, 40, or 60 g/kg body weight). Every morning, prior to ingesting the basal ration of alfalfa pellets, each lamb was offerend three foods for 15 min. The foods contained different amounts of energy and a toxin, depending on the addition of barley (energy) and LiCl (toxin) to alfalfa. The proportions of barley and LiCl changed every five days during the 25-day study. The results showed: (1) all lambs preferred food that was high > intermediate > low in energy (barley) in the absence of LiCl, but all lambs decreased consumption of foods high in energy as LiCl concentrations increased; (2) the greater the level of food deprivation, the lower the consumption of foods containing LiCl, even if the foods provided high levels of energy; (3) lambs moderately food deprived or fed ad libitum ingested more LiCl than lambs that were highly deprived; and (4) lambs quickly (15 min/day) regulated intake of foods in response to changes in barley and LiCl concentrations. Thus, our results suggest that the interaction between nutritional status and toxicosis plays an important role in food preference of lambs. Our findings also suggest that toxic plants may kill herbivores that lack nutritious alternative foods not only because the animals are forced to be less discriminating, but also because they are more susceptible to toxins.

Key Words—Food preference, nutrients, toxins, physiological condition, sheep, *Ovis aries*.

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INTRODUCTION

Herbivores face challenges in attempting to forage because plant chemistry changes constantly as a result of changes in the environment (McKey, 1979; Gershenzon, 1984; Harborne, 1991; Bryant et al., 1992). For instance, changes in weather (e.g., frost, cloudy weather) can alter levels of toxins and cause normally innocuous forages to become toxic (Everist, 1981). Water stress and nitrogen fertilization also increase concentrations of toxic compounds (e.g., cyanogenic glycosides, alkaloids, phenolics, terpenes) in some plant species (Gershenzon, 1984). Conversely, many plant species that grow in fertile soils and in shade contain lower levels of feeding-deterrent metabolites and higher levels of nutrients than plants that grow on infertile soils and in sunshine (Bryant et al., 1983). Thus, herbivores must select diets from foods whose toxin and nutrient concentrations fluctuate constantly.

In addition to changes in plant chemistry, an animal's nutritional needs also change throughout its lifetime in response to changes in its physiological condition, and from meal to meal depending on the nutrient content of the foods it ingests. Macronutrients (i.e., energy and protein) play a primary role in nutrition and food preference. For instance, lambs strongly prefer food high in readily fermentable energy, and they acquire strong preferences even for poorly nutritious foods like straw when straw is eaten during intraruminal infusions of energy (Villalba and Provenza, 1996). Nonetheless, their food preferences depend on what they just ate. For instance, lambs fed a basal ration high in energy (grain) subsequently prefer food lower in energy and higher in protein (alfalfa); those fed a ration high in protein (alfalfa) subsequently prefer food high in energy (grain) (Wang and Provenza, 1996). Their preferences also depend on how long it has been since they ate. When lambs are food deprived overnight, they prefer foods high in energy (barley), regardless of whether or not their basal ration is high in energy or protein (Wang and Provenza, 1996). Nonetheless, lamb's preference for barley over alfalfa decreases immediately (1 hr) after eating a meal of barley, and the more barley eaten, the greater the decrease in preference (Phy and Provenza, 1996). Thus, preference for food high in energy or protein depends on whether or not a lamb just ate a meal high in energy or protein.

Most studies treat nutrient needs and toxin avoidance as separate processes, but the interaction between these factors may play an important role in food preference. Animals may select toxic foods high in nutrients, or they may reject nutritious foods containing toxins, depending on their nutritional status. Herbivores often eat nutritious foods that contain toxins, and their intake is limited by their ability to detoxify or tolerate the toxin, which may be related to their nutritional state (Freeland and Janzen, 1974; Lindroth, 1988; McArthur et al., 1991; Smith, 1992; Foley et al., 1995; Illius and Jessop, 1995). Lambs regulate nutrient (Wang and Provenza, 1996) and toxin (Wang and Provenza, unpub-

lished data) intake based on their physiological condition, but little is known about how nutritional status interacts with toxicosis to affect food selection. Our objective was to assess the effects of different levels of food deprivation on the preferences of lambs for foods containing different amounts of energy and a toxin. Our primary interest was to learn if lambs fed alfalfa (which is high in protein but marginal in energy for growth) would ingest food high in energy, even if the food contained a toxin.

METHODS AND MATERIALS

The experiment was conducted during June of 1994 at the Green Canyon Ecology Center near Utah State University in Logan, Utah. Lambs (average body weight of 28 kg) were crosses of Columbia, Rambouillet, Suffolk, Targhee, and Finnish Landrace breeds. At 3 months of age, 30 lambs were moved to adjacent individual pens (1.3 m × 2.7 m in size), where they had access to water and mineral blocks ad libitum throughout the experiment.

Determining Ad Libitum Intake. For two weeks prior to the trials, all lambs were fed alfalfa pellets ad libitum and the daily intake (24 hr) of pellets by each lamb was measured. Lambs ate an average of 1700 g of alfalfa pellets daily [about 60 g/kg body weight (BW)/day].

Levels of Food Deprivation. We varied level of food deprivation by offering lambs (10 lambs/treatment) different amounts of alfalfa pellets (1) 20 g/kg BW/day (one third of ad libitum intake); (2) 40 g/kg BW/day (two thirds of ad libitum intake); or (3) 60 g/kg BW/day (ad libitum intake). Treatment 1 (20 g/kg BW/day) provided enough energy to meet lambs' requirements for maintenance, but not for growth (NRC, 1985). These three levels of deprivation were maintained throughout the 25-day trial.

Trials. Ten days after imposing the food-deprivation treatments, lambs were offered 400 g each of three foods. Barley and LiCl were added to alfalfa to create foods: (1) high, (2) intermediate, and (3) low in energy and a toxin, respectively (Tables 1 and 2). Barley and alfalfa were ground and mixed uniformly. The proportions of barley and LiCl in foods 1 and 2 changed every five days during the 25-day trial. All three foods always contained the same total concentrations of NaCl plus LiCl (NaCl tastes similar to LiCl) (Hill and Mistretta, 1990).

Lambs were offered the three foods from 08:00 to 08:15 hr daily. Intake of each food was measured immediately after each preference test. The amount of alfalfa pellets provided to each lamb for the remainder of the day was adjusted on the basis of energy intake of the test foods to maintain the level of food deprivation constant within treatments. The test foods were provided in three

TABLE 1. FOODS OFFERED TO LAMBS DURING 25-DAY EXPERIMENT

Days	Foods offered (15 min/day)
1–5	1. 50% alfalfa + 50% barley
	2. 75% alfalfa + 25% barley
	3. 100% alfalfa
6–10	1. 50% alfalfa + 50% barley + 2% LiCl
	2. 75% alfalfa + 25% barley + 1% LiCl + 1% NaCl
	3. 100% alfalfa + 2% NaCl
11–15	1. 25% alfalfa + 75% barley + 2% LiCl
	2. 50% alfalfa + 50% barley + 1% LiCl + 1% NaCl
	3. 100% alfalfa + 2% NaCl
16–20	1. 25% alfalfa + 75% barley + 3% LiCl
	2. 50% alfalfa + 50% barley + 1.5% LiCl + 1.5% NaCl
	3. 100% alfalfa + 3% NaCl
21–25	1. 25% alfalfa + 75% barley
	2. 50% alfalfa + 50% barley
	3. 100% alfalfa

adjacent food boxes attached to the wire panels that comprised each pen. We changed the positions of the three foods daily.

Statistical Analyses. The analysis of variance was a factorial with repeated-measures (Hicks, 1993). There were three treatments (20, 40, or 60 g/kg BW/day of alfalfa pellets) and three foods (high, intermediate, and low in energy and a toxin). Animals (10/treatment) nested within treatments was the error term used to test for differences among treatments. Animals/treatments × food was the error term used to test for differences in foods and to test the food × treatment interaction. Day (five days of tests within each period) was the repeated

TABLE 2. DIGESTIBLE ENERGY (DE) AND DIGESTIBLE PROTEIN (DP) CONCENTRATIONS OF FOODS USED IN EXPERIMENT[a]

Foods	DE (Mcal/kg)	DP (%)
100% alfalfa	2.3	10.9
75% alfalfa + 25% barley	2.5	10.6
50% alfalfa + 50% barley	2.8	10.4
25% alfalfa + 75% barley	3.1	10.1

[a]Calculated values for DE and DP are based on the following values obtained from Nutrient Requirements of Sheep (NRC, 1985): alfalfa 2.27 Mcal/kg DE, 10.9% DP; barley 3.35 Mcal/kg DE, 9.8% DP.

measure in the analyses. We separated the 25-day trial into five periods of five days each, based on the different foods offered, and analyzed each period separately using the SAS statistical program (Cody and Smith, 1991; Hatcher and Stepanski, 1994).

RESULTS

The largest fluctuations in intake of the three foods occurred in lambs receiving only 20 g/kg BW/day (Figure 1). In the absence of LiCl (days 1–5), preference was food 1 (high barley) > food 2 (medium barley) > food 3 (alfalfa) ($P < 0.001$). When LiCl was added to foods 1 and 2 (days 6–15) and then increased (days 16–20), preference changed to food 3 > 2 > 1 ($P < 0.01$ food × day interaction). When LiCl was removed from foods 1 and 2 (days 21–25), lambs' preference reverted to 1 > 2 > 3 ($P > 0.05$), and intake of foods 1 and 2 exceeded that on days 1–5, when barley content was less.

The response of lambs moderately food-deprived (40 g/kg BW/day) was intermediate to that of lambs fed 20 or 60 g/kg BW/day (Figure 2). In the absence of LiCl in the foods (days 1–5), food preference was food 1 (high

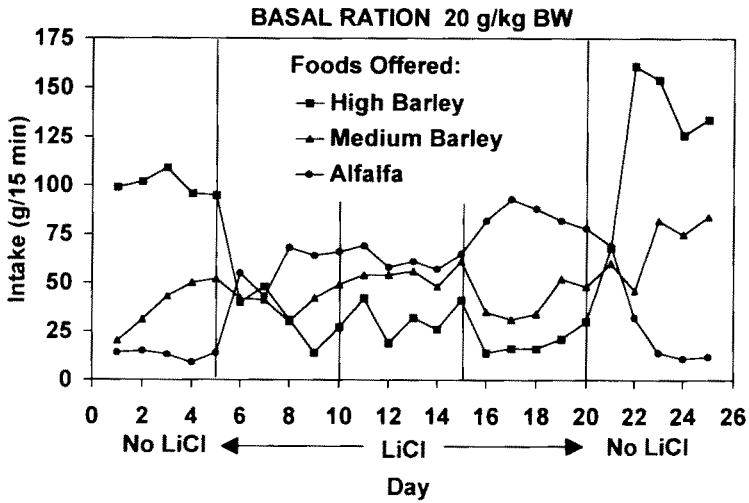

FIG. 1. Intake of three foods by lambs offered 20 g/kg BW/day of alfalfa pellets as a basal ration. Vertical lines represent changes in nutrient and toxin content of the three foods (see Table 1 for details). Standard errors were calculated for each feeding period: days 1–5 SEM = 6; days 6–10 SEM = 7; days 11–15 SEM = 8; days 16–20 SEM = 5; days 21–25 SEM = 10.

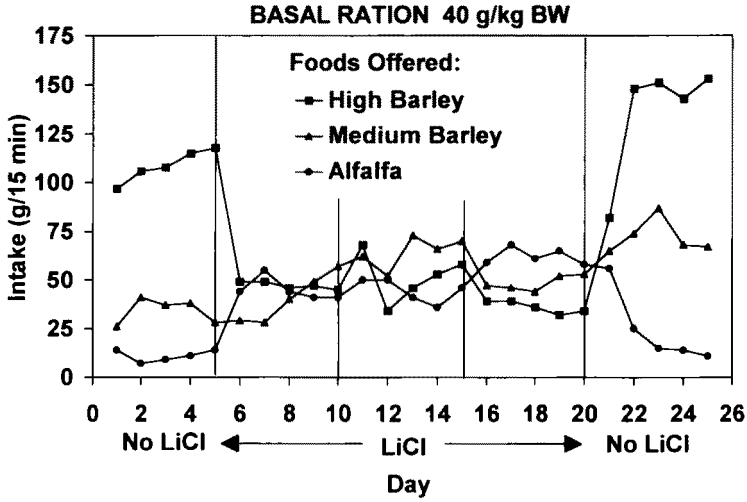

FIG. 2. Intake of three foods by lambs offered 40 g/kg BW/day of alfalfa pellets as a basal ration. Vertical lines represent changes in nutrient and toxin content of the three foods (see Table 1 for details). Standard errors were calculated for each feeding period: days 1–5 SEM = 6; days 6–10 SEM = 7; days 11–15 SEM = 8; days 16–20 SEM = 5; days 21–25 SEM = 10.

barley) > food 2 (medium barley) > food 3 (alfalfa) ($P < 0.01$). When LiCl was added to foods 1 and 2 (days 6–10), intake of food 1 decreased and intake of food 3 increased ($P < 0.01$ food by day interaction), and there was no difference in intake of the three foods ($P > 0.05$). Increasing barley concentrations (days 11–15) resulted in food preferences of 2 > 1 > 3 ($P > 0.01$ food by day interaction). A further increase in LiCl (days 16–20) resulted in a food preference of 3 > 2 > 1 ($P < 0.05$). Upon removal of LiCl (days 21–25), food preferences reverted to 1 > 2 > 3 ($P < 0.01$), and intake of foods 1 and 2 exceeded that on days 1–5, when barley constant was less.

Lambs with ad libitum access to alfalfa (60 g/kg BW/day) changed intake of the three foods when barley and LiCl concentrations changed (Figure 3). When foods contained no LiCl (days 1–5), food preferences were food 1 (high barley) > food 2 (medium barley) > food 3 (alfalfa) ($P < 0.01$). When LiCl was added to foods 1 and 2 (days 6–10), intake of food 1 decreased, intake of food 3 increased, and intake of food 2 remained the same ($P < 0.05$ food × day interaction). During days 11–15 when LiCl levels remained the same but barley concentrations increased in foods 1 and 2, intake of foods 2 and 3 increased, and intake of food 1 remained the same ($P < 0.01$ food × day interaction). Nevertheless, food preferences were still 1 > 2 > 3 (days 6–15,

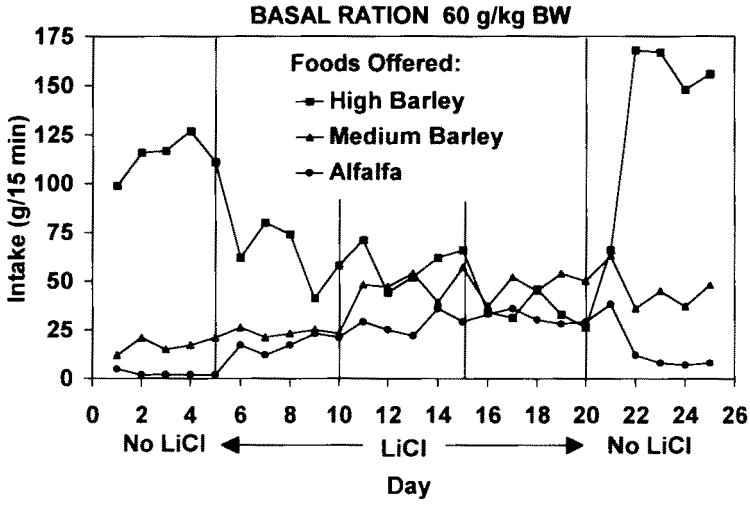

FIG. 3. Intake of three foods by lambs offered 60 g/kg BW/day of alfalfa pellets as a basal ration. Vertical lines represent changes in nutritent and toxin content of the three foods (see Table 1 for details). Standard errors were calculated for each feeding period: days 1-5 SEM = 6; days 6-10 SEM = 7; days 11-15 SEM = 8; days 16-20 SEM = 5; days 21-25 SEM = 10.

$P < 0.05$). When LiCl increased further in foods 1 and 2 (days 16-20), consumption of food 2 exceeded that of foods 1 and 3 ($P < 0.05$). When LiCl was removed from foods 1 and 2 (days 21-25), lambs' preference reverted to food 1 > 2 > 3, and intake of foods 1 and 2 exceeded that on days 1-5, when barley content was less.

Thus, food selection by lambs experiencing different levels of food deprivation can be summarized as follows. Intake of food 1 (high barley) did not differ among treatments when none of the foods contained LiCl (days 1-5, 21-25), but all lambs decreased intake when LiCl was added (days 6-20). However, consumption of food 1 was inversely related to level of food deprivation ($P < 0.05$). Lambs fed ad libitum ate less food 2 (medium barley) than lambs that were highly or moderately food deprived during days 1-10 and 21-25 ($P < 0.05$). All lambs ate more food 3 (alfalfa) during days 6-20 when LiCl was added to foods 1 and 2 ($P < 0.05$); consumption was most deprived > moderately deprived > not deprived ($P < 0.05$).

Lambs in the three treatments differed in their intake of LiCl when LiCl was added to foods 1 and 2 during days 6-20 ($P < 0.05$). Lambs highly deprived of food consumed less LiCl (40 mg/kg BW, SEM = 0.78) than lambs that were moderately deprived (57 mg/kg BW, SEM = 0.79) or fed ad libitum

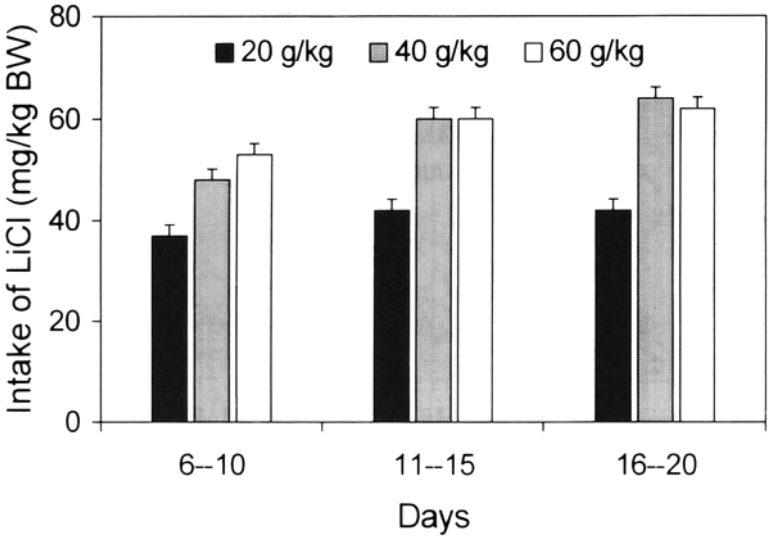

FIG. 4. Intake (SEM = 2.2) of LiCl by lambs offered 20, 40, or 60 g/kg BW of alfalfa as a basal ration during days 6–20 of the experiment.

(58 mg/kg BW, SEM = 0.77). Moreover, increasing barley levels enhanced intake of LiCl by lambs offered 40 or 60 g/kg BW/day of alfalfa. As barley concentrations were increased to 75% in food 1 and 50% in food 2 (from days 6–10 to days 11–15), the intake of LiCl increased from 48 to 60 mg/kg BW ($P < 0.05$) for lambs moderately deprived and from 53 to 60 mg/kg BW ($P < 0.05$) for lambs fed ad libitum (Figure 4). Lambs most food deprived increased intake of LiCl from 37 to 42 mg/kg BW, but the difference was not significant ($P > 0.05$). When the level of barley remained the same, but the level of LiCl increased in foods 1 and 2 (days 16–20), none of the lambs increased intake of LiCl (Figure 4).

DISCUSSION

Lambs quickly (15 min/day) regulated intake of foods in response to changes in the concentrations of energy (barley) and a toxin (LiCl). In the absence of LiCl, all lambs strongly preferred food high > intermediate > low in barley, and the higher the barley content of the food, the greater the preference (i.e., compare days 1–5 with days 20–25 for all treatments). The response to barley was expected because the lambs' basal ration of alfalfa was high in protein but marginal in energy for growth (Wang and Provenza, 1996). Nevertheless, despite

the strong preference for food high in energy, all lambs decreased consumption of foods high in energy as LiCl concentrations increased, and the greater the level of food deprivation, the lower the consumption of foods containing LiCl. Lambs most food deprived ingested lower levels of LiCl (40 mg/kg BW/day) than lambs that were moderately deprived (57 mg/kg BW/day) or fed ad libitum (58 mg/kg BW/day), in spite of the high concentration of barley (energy) in these foods. Thus, the interaction beween nutrient status and toxin avoidance apparently played an important role in food preference.

The adverse effects of food deprivation were evidently aggravated by the ingestion of LiCl because LiCl causes hypoglycemia [e.g., in rats and cattle (Johnson et al., 1980)]. This could explain why lambs fed only 20 g/kg BW of alfalfa pellets showed less preference for food containing LiCl, and it would also explain why increasing the barley content of the food (i.e., from days 6–10 to days 11–15) caused lambs fed 40 or 60 g/kg BW to increase their intake of LiCl. Inadequate nutrition can also decrease substrate needed by animals for detoxification processes (McArthur et al., 1991), and the maximum absorbed allelochemical dose may depend on the supply of cosubstrate for conjugation (Foley et al., 1995; Illius and Jessop, 1995). For instance, a reduction in the sulfur-containing amino acids methionine and cysteine can reduce serum inorganic sulfate in rats and reduce acetaminophen conjugation with sulfate (Glazenberg et al., 1983). Fasting increases acetaminophen toxicity in rats, as glucuronidation and sulfate formation are reduced (Price et al., 1987).

Managers often attempt to reduce livestock losses by preventing food-deprived animals from grazing areas that contain poisonous plants, based on the assumption that food-deprived animals are more likely to consume toxic doses of poisonous plants (Krueger and Sharp, 1978; Stoddart et al., 1975; Dwyer, 1978; Ralphs and Sharp, 1987; James et al., 1992). It is also possible that food-deprived animals are more susceptible to the effects of toxins (Foley et al., 1995; Illius and Jossop, 1995). Thus, a level of toxin that may be safe for satiated animals may not be safe for animals that are food deprived.

Many believe food-deprived animals select foods less discriminately and are more likely to overingest poisonous plants. Our results suggest this is not likely to occur if other foods are present, even in limited amounts. Food-deprived lambs strongly preferred foods high in energy (barley), but they avoided foods high in barley when they contained LiCl. Cattle also ingest less tall larkspur, a serious poisonous plant on mountain rangelands in the western United States, with decreasing levels of rumen fill (Pfister et al., 1988).

Several theories seek to explain how herbivores track changes in nutrients and toxins in variable environments (Provenza and Balph, 1990). Some postulate that animals associate food flavor (odor and taste) with postingestive feedback (Garcia, 1989; Provenza et al., 1992; Provenza, 1995, 1996), which enables animals to experience the consequences of food ingestion, thereby determining

preferences for flavors. On this basis, lambs may have identified changes in the flavor intensity of barley, alfalfa, and LiCl, which they associated with positive and aversive postingestive effects from nutrients and a toxin. Flavor-feedback relationships thus may enable animals to recognize changes in nutrients and toxins, thereby allowing them to adjust intake accordingly.

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VARIATION OF TOTAL PHENOLIC CONTENT AND INDIVIDUAL LOW-MOLECULAR-WEIGHT PHENOLICS IN FOLIAGE OF MOUNTAIN BIRCH TREES (*Betula pubescens* ssp. *tortuosa*)

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Abstract—We studied seasonal and between-tree variation in the composition and content of total and individual low-molecular-weight phenolics (LMWP) in leaves of mountain birch trees (*Betula pubescens* ssp. *tortuosa*). The major phenolic compounds were chlorogenic acid, quercetin-3-*O*-β-D-glucuronopyranoside, myricetin-3-*O*-(5-acetyl)-L-rhamnopyranoside, and 1-*O*-galloyl-β-D-(2-*O*-acetyl)-glucopyranose. The content of total phenolics, as well as the sum of individual LMWP, varied only slightly among trees while variation in contents of individual LMWP was large. Concentrations of almost all phenolics decreased during the growing season but pairwise correlations between individual phenolics remained similar over the whole season indicating tree-specific LMWP profiles over the season. Among flavonoids, the between-tree component of variation was 2.6 times as large as the seasonal component, while for variation of nonflavonoids the between-tree component was larger than the seasonal one. To explain the significant correlations within both flavonoid and nonflavonoid compounds, we discuss the biogenesis of LMWP in birch leaves, as well as their ecological role.

Key Words—*Betula pubescens* ssp. *tortuosa*; birch; phenolics, flavonoid glycosides; among-tree variation; seasonal variation.

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INTRODUCTION

The value of host plant tissue for herbivores is a function of its biochemical composition and physiological state of the plant. General theories of plant defenses share the basic assumption that secondary compounds are responsible for plant resistance, including induced defenses, and that these, rather than nutritive factors, represent the critical chemical factors explaining variance in herbivore performance (but see, e.g., Haukioja et al., 1991; Berenbaum, 1995). Plant phenolics are known for their negative effects on insects and they have played an important role in theories of plant–herbivore interactions (Feeny, 1976; Rhoades and Cates, 1976; Coley et al., 1985), and they are the defensive compounds generally measured from plant tissue.

However, in addition to their possible defensive role against herbivores, phenolics in plants have a suite of other possible functions, including antidesiccation, UV protection, growth regulation, etc. (Kuiters, 1989; Li et al., 1993; Lois, 1994; Northup et al., 1995). Furthermore, they seem to accumulate in slowly growing plants and plant tissues. The original idea of all phenolics having a basically similar and nonspecific effect on herbivores via protein precipitation (Feeny, 1976; Schultz, 1989) is far too simple. Different phenolics can vary in their biological activity, and in the mechanism of action on herbivores. Some phenolics may have a direct toxic effect on herbivores or require oxidation for realization of their ecological activity (Felton et al., 1989; Appel, 1993).

Total phenolics in birch leaves, and in many other plants, correlate negatively with insect performance (Haukioja et al., 1985). Furthermore, it is known that total phenolics may experience strong seasonal patterns, but what happens among individual phenolics is not well understood. We have recently identified 33 low-molecular-weight phenolics (LMWP) in the leaves of white (*Betula pubescens*) and silver (*B. pendula*) birches (Ossipov et al., 1995, 1996) by preparative HPLC and determined their structures on the basis of chromatographic (analytical HPLC), chemical (hydrolysis), and spectroscopic (UV, MS, ^{1}H NMR and ^{13}C NMR) techniques. In this paper we examine the composition and content of low-molecular-weight phenolics in leaves of individual mountain birch trees over a season to find out how well total phenolic deteminations describe seasonal and between-tree variation in the contents of individual low-molecular-weight phenolics of the leaves of mountain birch.

METHODS AND MATERIALS

Plant Material. The experimental mountain birches [*Betula pubescens* Ehrh ssp. *tortuosa* (Lebed.) Nyman] were mature, wild trees growing near the Kevo

Subarctic Research Station (69°45'N, 27°01'E) in northern Finland. The study area lies at about 150 m above sea level and is covered with almost pure mountain birch forest. We sampled foliage of the same 20 birch trees that were originally randomly chosen from a homogeneous forest plot and marked individually by Hanhimäki et al. (1996).

Short shoot leaves from individual trees were sampled on July 1 and 20, and August 24, 1994, always between 9 and 10 AM. Leaves even at the earliest sampling date were almost full grown. Sampling dates were designed to coincide with the peak feeding of the two most important insect herbivores of the mountain birch, the autumnal moth, *Epirrita autumnata* (early July) and the sawfly, *Dineura virididorsata* (August), as well as to include an intermediate date when many mid-season sawflies consume foliage (Hanhimäki et al., 1995). Since the short shoot leaves of the mountain birch burst simultaneously in spring, all the leaves within a tree were of the same age on a given sampling date and matured similarly between the sampling dates. Excised leaves were placed in plastic vials and returned to the laboratory on ice. For determination of phenolic compounds, the birch leaves were vacuum dried and stored in plastic vials at −20°C.

Extraction of Phenolics. The dried leaves (0.5 g) were ground for 2 min with Ultra-turrax T 25 in 25 ml of 70% aqueous acetone. The homogenate was centrifugated at 2500*g* for 10 min and the pellet was reextracted with 25 ml of 70% aqueous acetone for 60 min under continuous stirring. After centrifugation the combined extracts were evaporated to dryness under low pressure at 40°C. The residue was dissolved in 10 ml of water and centrifuged. Then the solution was filled to 25 ml of water and stored in a freezer (−20°C). Extraction efficiency of phenolic compounds was confirmed by reextracting the pellet three times with 25 ml of 70% aqueous acetone and checking these solutions by HPLC.

Total Content of Phenolics. A modification of the Folin-Ciocalteau method (Torres et al., 1987) was used for measurement of total content of phenolic compounds. A 0.1-ml extract was delivered to a 15-ml tube and mixed with 5.9 ml water. One milliliter of this diluted extract was mixed with 1.0 ml 1 N Folin reagent (Folin-Ciocalteau's phenol reagent, Fluka, BioChemica, Buchs, Switzerland) in the centrifuge tube (10 ml volume) and the mixture was allowed to stand for 2–5 min. Then 2 ml of 20% Na_2CO_3 was added. After a 10-min incubation at room temperature, the mixture was centrifuged at 1500*g* for 8 min and the absorbance was measured at 730 nm on a Perkin-Elmer Spectrophotometer 550. Three replicates of each sample were analyzed. The standard curve was prepared with known concentrations of gallic acid.

Quantification of LMWP. Before the HPLC analysis of phenolics, the extract was filtered through Millex-HV_{13} 0.45-μm filter (Millipore, Bedford, Massachusetts). The HPLC system consisted of Merck-Hitachi Pump L-6200 A,

Merck-Hitachi L-4250 UV-VIS Detector (Hitachi, Tokyo, Japan), Perkin-Elmer LC-235 Diode Array Detector (Shimadzu, Kyoto, Japan), and Shimadzu C-R6A Chromatopac Integrator (Shimadzu, Kyoto, Japan). The sample was injected on Spherisorb ODS-2 column (250 × 4.6 mm ID, 5 μm; Phase Sep. Ltd.) and the flow rate was 1.0 ml/min. Detection was performed at 280 nm, 0–40 min; 360 nm, 40–60 min; and 280 nm, 60–110 min. Two solvents were used; solvent A was 5% formic acid and solvent B was acetonitrile. The gradient was as follows: 0–5 min, 100% A (Isocratic); 5–60 min, 0–30% B in A (linear gradient); 60–70 min, 30–60% B in A (linear gradient); 70–80 min, 60% B in A (Isocratic). Quantitative determinations were carried out by the external standard method using gallic, *p*-coumaric, and chlorogenic acids, quercetin, myricetin, and kaempferol as standards, assuming a similar molecular extinction coefficient for aglycones and glycosides (Winter and Herrmann, 1986).

The relative standard error in the quantitation of LMWP, ranging from 2.2% to 11.2% (small peaks) based on HPLC analysis of five leaf samples of the same tree, provides an estimation of the error involved.

Statistical Analysis. The distributions of a few phenolic compounds deviated from the normal. The homogeneity of variances was tested with Cochran's test; for a few phenolic compounds, the variances differed significantly. There were, however, no deviations from normality or homoscedasticity with these compounds. Significance of variations in the content of phenolic compounds were tested by a two-way ANOVA. The correlations between individual phenolic compounds during the growing season were tested with Pearson's correlation analysis (Proc Corr, SAS Institute, Cary, North Carolina).

RESULTS

Identification. Up to 30 individual low-molecular-weight phenolic compounds in the leaves of the mountain birch were detected by HPLC (Figure 1). Composition of LMWP in the leaves of mountain birch were closely consistent with our earlier analyses of white and silver birch leaf chemistry (Ossipov et al., 1995, 1996). The identified phenolics included both flavonoid (peaks 4, 5, 11, 13, 20–36) and nonflavonoid (peaks 1, 2, 6, 8, 9, 12, 14, 18, 19) compounds.

We detected 21 flavonoids and identified 11 of them on the basis of UV spectroscopy data as flavanols (peaks 4, 5, 13, 20–22, 25), quercetin glycosides (30, 32), or kaempferol glycosides (35, 36). The identified flavonoid compounds were the glycosides of *m*yricetin, M-3-*O*-β-D-glucuronopyranoside (23), M-3-*O*-β-D-galactopyranoside (24), and M-3-*O*-L-(acetyl)-rhamnopyranoside (26); glycosides of quercetin, Q-3-*O*-β-D-glucuronopyranoside (27), Q-3-*O*-β-D-galactopyranoside (28), Q-3-*O*-α-L-arabinofuranoside (29), Q-3-*O*-α-L-(4″-*O*-ace-

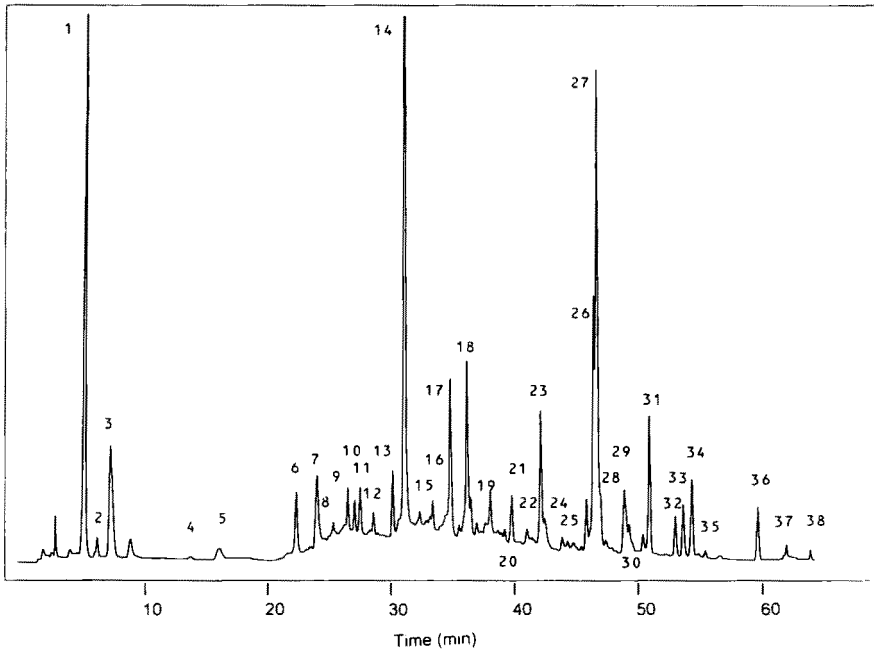

FIG. 1. Typical trace of HPLC analysis of soluble low-molecular-weight phenolics (1–40) in the leaves of mountain birch trees. Spherisorb ODS-2 column (250 × 4.6 mm ID, 5 μm) at a flow rate of 1.0 ml/min with gradient of acetonitrile in 5% aqueous formic acid; detection wavelength at 280 nm. Peak 1, 1-*O*-galloyl-β-D-(2-acetyl)-glucopyranose; 2, gallic acid; 4 and 5, flavanois; 6, neochlorogenic acid; 8, *cis*-5-*p*-coumaroylquinic acid; 9, *trans*-5-*p*-coumaroylquinic acid; 11, (+)-catechin; 12, 1-(4″-hydroxyphenyl)-3′-oxopropyl-β-D-glucopyranose; 13, flavanol; 14, chlorogenic acid; 18, *trans*-3-*p*-coumaroylquinic acid; 19, *cis*-3-*p*-coumaroylquinic acid; 20–22, flavanols; 23, myricetin-3-*O*-β-D-glucuronopyranoside; 24, myricetin-3-*O*-β-D-galactopyranoside; 25, flavanol; 26, myricetin-3-*O*-α-L-(acetyl)-rhamnopyranoside; 27, quercetin-3-*O*-β-D-glucuronopyranoside; 28, quercetin-3-*O*-β-D-galactopyranoside; 29, quercetin-3-*O*-α-L-arabinofuranoside; 30, quercetin glycoside; 31, kaempferol-3-*O*-β-D-glucopyranoside; 32, quercetin glycoside; 33, kaempferol-3-*O*-α-L-rhamnopyranoside; 34, quercetin-3-*O*-α-L-(4″-*O*-acetyl)-rhamnopyranoside; 35 and 36, kaempferol glycosides. Peaks 3, 7, 10, 15, 16, 17, 37, and 38 were not identified.

tyl)-rhamnopyranoside (34); and glycosides of kaempferol, K-3-*O*-β-D-glucopyranoside (31) and K-3-*O*-α-L-rhamnopyranoside (33). Compound 11 was identified as the flavanol, (+)-catechin.

The identified nonflavonoid compounds were gallic acid (peak 2) and its derivative, 1-*O*-galloyl-β-D-(2-*O*-acetyl)-glucopyranose (1); derivatives of caf-

feic acid, neochlorogenic acid (6, *trans*-5-p-caffeoylquinic acid) and chlorogenic acid (14, *trans*-3-caffeoylquinic acid); and derivatives of *p*-coumaric acid, *cis*-5-p-coumaroylquinic (8), *trans*-5-*p*-coumaroylquinic (9), *trans*-3-*p*-coumaroylquinic (18), and *cis*-3-*p*-coumaroylquinic (19) acids. Peak 12 was identified as 1-(4″-hydroxyphenyl)-3′-oxopropyl-β-D-glucopyranose.

Seasonal Variation. The mean total phenolics content in the leaves of mountain birch in early, mid-, and late summer was 103, 93, and 89 mg/g of dry wt, respectively; the average decline from early July to late August was 14%.

The mean content of the main individual low-molecular-weight phenolics and total content of phenolics in the leaves of the 20 trees used in our study are shown in Table 1. For quantitative calculations, the main 18 phenolic compounds were considered; the remaining 12 compounds (peaks 4, 5, 8, 9, 11–13, 20–22, 25, 35, 37 and 38) were disregarded due to lower levels of accumulation and poor separability of the respective peaks. Most phenolic parameters showed significant differences among sampling dates (Table 2).

The mean total LMWP content of foliage in early, mid-, and late summer was approximately 48, 41, and 38 mg/g of dry wt, respectively. The average decline was 21%, i.e., steeper than in the case of total phenolics. The average seasonal decline of nonflavonoid compounds ranged from 52% (6) to 70% (19), except for peak 14, the average content of which increased 12% in July, followed by a 15% drop in late August. The content of individual flavonoid compounds declined at the same time from 7% (28) to 37% (23). The amount of most phenolic compounds declined evenly during the three sampling dates, but compound 1 declined rapidly in July, over 80% of total decrease. The average content of flavonoid compounds 26, 28, and 30, however, increased over 10% in late summer compared to mid-summer.

Between-Tree Variation. Differences in the composition of LMWP between the 20 trees were small, and most compounds could be detected from each tree. However, compounds 32, 34, and 36 were found in less than one third of the trees, and compounds 1, 2, 6, and 30 occurred in small or trace amounts in some of the trees.

The quantitative variation in phenolic compounds was large throughout the growing season (Table 1). Between-tree variation was significant at $P < 0.001$ in all phenolics through the growing season, except for gallic acid (peak 2). In early summer the coefficients of between-tree variation (CV) of nonflavonoid compounds ranged from 42% (19) to 93% (6), and of flavonoid compounds from 36% (27) to 264% (36), being less than 70% for two thirds of the compounds. The tendency was similar throughout the growing season. Still, the coefficient of between-tree variation of total content of LMWP (the sum of individual compounds) and total phenolics varied during the season only between 23 and 27% and 14 and 17%, respectively.

TABLE 1. CONTENT OF LOW-MOLECULAR-WEIGHT PHENOLICS AND TOTAL PHENOLICS IN LEAVES OF 20 MOUNTAIN BIRCH TREES ON THE THREE SAMPLING DATES (EARLY, MID, AND LATE SEASON)[a]

Peak[b]	Compound	Sampling date			Drop (%)
		Early	Mid	Late	
1	1-*O*-galloyl-D-(2-ac.)-glucopyranoside	3.70 ± 3.16	1.80 ± 1.56	1.36 ± 1.45	63
2	gallic acid	0.26 ± 0.17	0.05 ± 0.03	0.01 ± 0.00	100
6	*trans*-5-caffeoylquinic acid	1.03 ± 0.96	0.67 ± 0.66	0.49 ± 0.73	52
14	chlorogenic acid	13.74 ± 8.26	15.36 ± 9.74	12.98 ± 8.12	6
18	*trans*-3-*p*-coumaroylaquinic acid	1.36 ± 0.80	0.73 ± 0.33	0.41 ± 0.11	70
19	*cis*-3-*p*-coumaroylquinic acid	0.35 ± 0.15	0.25 ± 0.11	0.13 ± 0.03	62
23	M-3-*O*-D-glucuronopyranoside	2.72 ± 1.32	2.15 ± 0.85	1.71 ± 0.66	37
24	M-3-*O*-D-galactopyranoside	0.71 ± 0.43	0.60 ± 0.33	0.53 ± 0.35	25
26	M-3-*O*-(5-ac.)-L-rhamnopyranoside	5.17 ± 2.75	3.99 ± 1.99	4.66 ± 1.98	10
27	Q-3-*O*-D-glucuronopyranoside	9.58 ± 3.47	8.14 ± 3.07	8.23 ± 3.03	14
28	Q-3-*O*-galactopyranoside	1.88 ± 1.09	1.54 ± 0.91	1.76 ± 1.03	7
29	Q-3-*O*-α-L-arabinofuranoside	2.11 ± 1.10	1.68 ± 0.86	1.48 ± 0.83	30
30	Quercetin-glycoside[c]	0.73 ± 0.78	0.54 ± 0.65	0.60 ± 0.62	17
31	K-3-*O*-D-glucopyranoside	2.02 ± 0.99	1.52 ± 0.79	1.56 ± 0.85	23
32	Quercetin glycoside[c]	0.34 ± 0.53	0.32 ± 0.49	0.27 ± 0.41	18
33	K-3-*O*-L-rhamnopyranoside	1.19 ± 0.63	0.93 ± 0.49	0.82 ± 0.44	31
34	Q-3-*O*-α-L-(4″-*O*-ac.)-rhamnopyranoside	0.65 ± 1.37	0.56 ± 1.14	0.50 ± 1.04	24
36	Kaempferol glycoside[d]	0.11 ± 0.30	0.11 ± 0.23	0.09 ± 0.20	20
	Total LMWP	47.64 ± 10.87	40.93 ± 11.16	37.58 ± 9.69	21
	Total phenolics	103.12 ± 16.68	92.63 ± 13.06	88.50 ± 14.89	14

[a] Values are mg/g dry weight (mean ± 1 SD). Q, quercetin; K, kaempferol; M, myricetin.
[b] Refers to peak numbers in Figure 1.
[c] Expressed as quercetin-3-*O*-D-rhamnopyranoside (M = 448.16 g/mol).
[d] Expressed as kaempferol-3-*O*-D-glucopyranoside (M = 448.36 g/mol).

TABLE 2. RESULTS OF TWO-WAY ANOVA WITHOUT REPLICATION FOR DIFFERENCES IN CONTENTS OF LOW-MOLECULAR-WEIGHT PHENOLICS AND TOTAL PHENOLICS OF LEAVES OF 20 MOUNTAIN BIRCHES DURING THE SEASON.

	Among trees		Temporal variation	
Peak[a]	MS	F	MS	F
1	11.99	9.49***[b]	3071	24.29***
2	0.01	1.22	0.38	39.23***
6	1.64	13.29***	1.48	12.02***
14	219.66	46.87***	29.52	6.30**
18	0.48	3.36***	4.68	32.90***
19	0.02	4.11***	0.23	41.12***
23	2.56	15.08***	5.13	30.31***
24	0.38	25.68***	0.16	10.67***
26	14.33	25.51***	7.02	12.49***
27	30.06	94.96***	12.99	41.03***
28	3.01	78.53***	0.59	15.50***
29	1.38	74.89***	0.18	9.56***
30	2.49	37.45***	2.05	30.91***
31	2.25	61.78***	1.53	42.12***
32	0.67	72.72***	0.02	2.29
33	0.79	40.52***	0.74	37.82***
34	4.20	111.17***	0.12	3.29*
36	0.16	15.81***	<0.01	0.25
Total LMWP	316.61	31.99***	524.49	53.00***
Total phenolics	610.88	20.43***	1135.93	37.99***

[a] Refers to peak numbers in Figure 1.
[b] *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

The content of the major individual LMWP of mountain birch, chlorogenic acid (peak 14), varied among trees between 4.6 and 31.1 mg/g of dry wt (CV = 60%) in early summer. At the same time the content of other main nonflavonoid compounds (1, 6, 18, and 19) ranged from 0.1 to 1.5 mg/g of dry wt, except compound 1, which varied from 0.1 to 12.0 mg/g of dry wt (CV = 85%).

The main flavonoid compounds of mountain birch are quercetin-3-*O*-β-D-glucuronopyranoside (peak 27), myricetin-3-*O*-(5′-ac.)-L-rhamnopyranoside (26) and myricetin-3-*O*-β-D-glucuronopyranoside (23), the contents of which varied in early summer between 3.9 and 16.5 (CV = 36%), 1.2 and 10.5 (CV = 53%), and 0.8 and 6.2 (CV = 48%) mg/g of dry wt, respectively. The main kaempferol glycoside of mountain birch is compound 31, K-3-*O*-β-D-glucopy-

TABLE 3. PAIRWISE TREE-SPECIFIC CORRELATIONS BETWEEN LOW-MOLECULAR-WEIGHT PHENOLICS (NONFLAVONOIDS) OF LEAVES OF 20 MOUNTAIN BIRCHES STUDIED IN EARLY, MID- AND LATE SUMMER

		Peak				
Peak[a]	Date	1	2	6	14	18
2	early	0.80***[b]				
	mid	0.67**				
	late					
6	early	0.41	0.34			
	mid	0.20	0.31			
	late	0.13				
14	early	0.03	0.21	−0.08		
	mid	0.08	0.00	−0.24		
	late	0.31		−0.20		
18	early	0.08	0.29	0.04	0.84***	
	mid	0.06	−0.20	−0.29	0.71***	
	late	0.32		−0.07	0.59**	
19	early	0.11	0.40	0.00	0.78***	0.92***
	mid	0.10	−0.09	−0.27	0.73***	0.94***
	late	−0.22		−0.02	−0.16	0.33

[a]Refers to peak numbers in Figure 1.
[b]*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

ranoside, whose amounts varied between 0.8 and 4.1 mg/g of dry wt (CV = 49%).

Intraspecific Relationships of LMWP. Intraspecific relationships of phenolic compounds are shown in Tables 3 and 4 (not all the correlations are shown). Tree-specific correlations between most individual phenolic compounds remained similar over the whole season.

The amounts of nonflavonoid compounds (peaks 14, 18, and 19) correlated positively ($P < 0.01$) over the growing season, except for compound 19 in late summer samples. These compounds also correlated positively with total LMWP content, e.g., correlation coefficients of compound 14 with total content of LMWP varied between 0.87 and 0.95 ($P < 0.001$) over the whole season. The amounts of gallic acid (2) and the acetylated form of β-glucogallin (1) correlated positively ($P < 0.001$) in early and mid-summer samples.

The main flavonoid compound, quercetin-3-*O*-β-D-glucuronopyranoside (27) correlated positively, but weakly, with quercetin-glycosides (28 and 29), and negatively with compounds 30 and 34 (Table 4). Between quercetin gly-

TABLE 4. THE PAIRWISE TREE-SPECIFIC CORRELATIONS OF FLAVONOIDS IN LEAVES OF 20 MOUNTAIN BIRCHES STUDIED AT EARLY, MID-, AND LATE SUMMER

		Peak										
Peak[a]	Date	23	24	26	27	28	29	30	31	32	33	34
24	early	0.46										
	mid	0.45*[b]										
	late	0.45**										
26	early	0.30	−0.25									
	mid	0.10	−0.46									
	late	0.01	−0.49*									
27	early	−0.10	−0.04	−0.49*								
	mid	−0.11	0.05	−0.51*								
	late	−0.17	0.06	−0.32								
28	early	−0.17	0.60**	−0.50*	0.41							
	mid	−0.11	0.61**	−0.55*	0.48*							
	late	−0.13	0.60**	−0.45*	0.51*							
29	early	−0.14	0.40	−0.29	0.35	0.66**						
	mid	−0.07	0.52*	−0.50*	0.40	0.53*						
	late	−0.08	0.54*	−0.42	0.39	0.55*						
30	early	0.10	−0.30	0.76***	−0.56*	−0.53*	−0.22					
	mid	−0.11	−0.39	0.79***	−0.51*	−0.45*	−0.28					
	late	−0.06	−0.39	0.74***	−0.48*	−0.48*	−0.30					
31	early	−0.31	−0.33	0.07	0.27	0.09	0.03	0.17				
	mid	−0.54	−0.31	0.23	0.21	0.12	0.05	0.41				
	late	−0.53**	−0.27	0.40	0.21	0.17	0.03	0.46*				

32	early	0.18	−0.22	−0.05	−0.34	−0.46*	−0.28	0.15	−0.23			
	mid	0.06	−0.30	0.00	−0.26	−0.45*	−0.30	0.03	−0.32			
	late	0.07	−0.29	−0.02	−0.26	−0.44	−0.42	0.07	−0.31			
33	early	−0.32	0.23	−0.14	0.02	0.56**	0.63**	0.04	0.56*	−0.15		
	mid	−0.47*	0.21	−0.07	−0.04	0.43	0.56**	0.25	0.62**	−0.22		
	late	−0.45*	0.17	0.07	−0.02	0.43	0.50*	0.31	0.68***	−0.27		
34	early	0.13	−0.02	−0.08	−0.49	−0.24	−0.09	0.21	−0.17	0.83***	0.09	
	mid	−0.04	−0.06	−0.09	−0.51*	−0.21	−0.18	0.12	−0.30	0.72***	0.04	
	late	−0.04	−0.12	−0.16	−0.53*	−0.25	−0.29	0.13	−0.25	0.67**	0.01	
36	early	0.21	0.02	0.00	−0.25	−0.14	0.09	0.10	0.06	0.72***	0.27	0.80***
	mid	−0.03	−0.02	−0.05	−0.49*	−0.16	−0.15	0.10	−0.19	0.70***	0.15	0.93***
	late	0.03	−0.08	−0.14	−0.54*	−0.25	−0.34	0.17	−0.22	0.66**	0.01	0.98***

[a] Refers to peak numbers in Figure 1.
[b] $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

cosides (32 and 34) and kaempferol glycoside (36) the correlations were strongly positive ($P < 0.01$), as between kaempferol glycosides (31 and 33), and myricetin glycosides (23 and 24) ($P < 0.01$) (Table 4). Myricetin-3-*O*-β-D-galactopyranoside (24) and quercetin-3-*O*-β-D-galactopyranoside (28) correlated also positively through the growing season.

DISCUSSION

Understanding the importance of correlations between the concentrations of individual LMWP presumes understanding the biogenesis of these compounds. Figure 2 shows a simplified scheme of biosynthesis of the phenolics in woody plants that may be extended to birch.

The original substance for the synthesis of phenolic compounds in plants is phenylalanine, one of the end products of the shikimate pathway. Only gallic and protocatechuic acids are formed from an intermediate compound of the shikimate pathway—from dehydroshikimic acid (Gross, 1992). The positive correlation between the contents of gallic acid (2) and the acetylated form of β-glucogallin (1) in birch leaves is due to the presence of biosynthetic connections between these phenolics: gallic acid is a precursor of acetylglucogallin. Moreover, the low concentration of gallic acid indicates that its formation is a limiting step on the pathway of acetylglucogallin synthesis.

The biosynthesis of chlorogenic acid is possible in higher plants via *p*-coumaroyl or caffeoyl derivatives. Both routes are realized in different plants (Zenk, 1979). However, the positive correlation between the contents of chlorogenic (14) and *cis*- and *trans*-3-*p*-coumaroylquinic acids (18, 19) supports our assumption that in the birch leaves the biosynthetic pathway involves reactions of *p*-coumaroyl-CoA esterification by quinic acid and hydroxylation of 3-*p*-coumaroylquinic acid to chlorogenic acid (Figure 2).

Unlike in many other woody plants, in which the concentration of quinic acid may reach 10–12% of dry weight (Ossipov and Shein, 1990), birch leaves did not accumulate quinic acid, apparently due to its active transformation to 3-*p*-coumaroylquinic acids and especially to chlorogenic acid, the concentration of the latter being as high as 4% of dry weight in some trees. In addition, the high correlation coefficient between *trans*- and *cis*-3-*p*-coumaroylquinic acids (0.92–0.94, $P < 0.001$) supports our assumption that the formation of the *cis* form in the birch leaves is an enzymatic process that occurs under intercellular control (Ossipov et al., 1996).

There are four main groups of flavonoids in the birch leaves: the kaempferol, quercetin, and myricetin glycosides and the flavanols. Flavonoids of these groups correlated with each other differently. Correlations between glycosides within the same group and between glycosides of different groups varied from

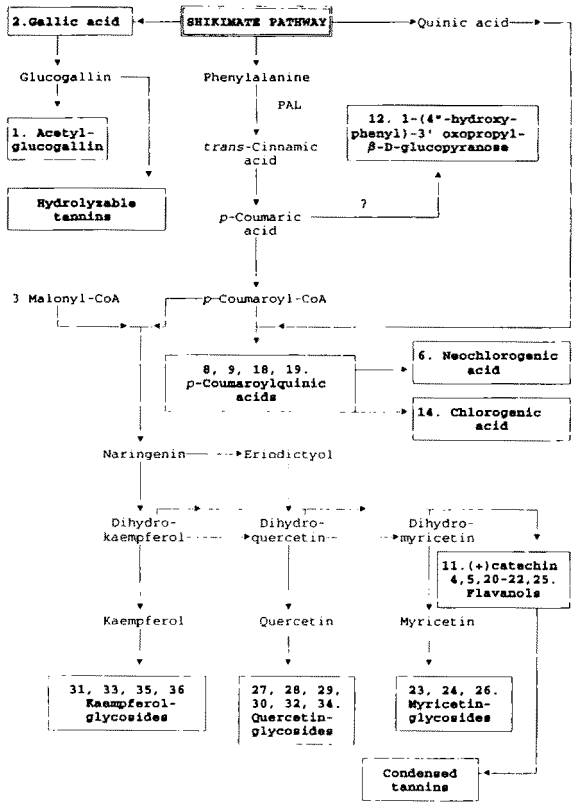

FIG. 2. Biogenesis of birch phenolics.

positive and negative (Table 4). However, the pathway of flavonoid synthesis involves a number of intermediates. Moreover, some of the flavonoids are not the end products of metabolism. In birch leaves they can be used in the biosynthesis of polymeric compounds.

Seasonal Variation. Total phenolic content has been shown to increase through the growing season in foliage of many perennial plant species (Feeny, 1970; Dement and Mooney, 1974; Lawton, 1976; Cooper-Driver et al., 1977), but no strong seasonal trends have been found in others (Macauley and Fox, 1980, Schultz et al., 1982, Baldwin et al., 1987).

In the mountain birch, the contents of both total phenolics and individual LMWP declined towards the end of growing season. The seasonal decrease in the phenolic contents may result from three main causes: (1) decreasing rate of their biosynthesis, (2) conversion into insoluble cell wall bound components,

and (3) active transformation into oligo- and polymeric compounds, for example, tannins or lignins. Moreover, this may be partly due to a dilution effect because of an increasing relative content of cell-wall components in the leaf; cellulose, hemicellulose, pectin, and lignin.

In our case, since we sampled even-aged short shoot leaves only, aging of leaves was important for the decrease of phenolics. Basically similar or variable seasonal trends have been shown with phenolic glycosides in the leaves of *Populus* (Thieme, 1971, Lindroth et al., 1987), and of *Salix* (Thieme, 1965, 1971, Julkunen-Tiitto, 1989).

Between-Tree Variation. Earlier, Haukioja et al. (1985) showed that the coefficient of variation in total phenolics among mountain birch trees ranged from 12 to 24%, which corresponds well to the results of the present study. Similarly, coefficients of variation of different phenolic compounds among trees in our study were about the same as those found by Suomela et al. (1995). Similar results were obtained with phenolic variation in the bark of winter-dormant seedlings of *Betula pendula* of several origins (Tahvanainen et al., 1991), and in leaves of several *Artemisia* species (Wilt et al., 1992).

When comparing between- and within-tree variation of LMWP for randomly sampled same-aged mountain birch trees, Suomela et al., (1995) showed that trees accounted, in general, for most of the total variance in different foliage phenolics. In 24 of the 27 components, trees accounted for over 75% (in most cases over 90%) of the total variance.

Our results contrast with flavonoid variation in *Chondrapetalum*. Harborne et al. (1985) found that while different collections of *C. microcarpum* had identical flavonol glycoside patterns and the same percentage of total flavonoids, the relative amounts of all the compounds (simple glycosides of myricetin, laricitrin, and syringenin) differed. In mountain birch, amounts of total LMWP and total phenolics differed among trees only slightly compared to variation in individual phenolic compounds.

Seasonal Versus Between-Tree Variation. Our results of seasonal and between-tree variations in mountain birch foliage through the growing season are consistent with the variability in *Artemisia* species (Wilt et al., 1992; Wilt and Miller, 1992). In mountain birch, the relative importance of seasonal and between-tree variations changed with different phenolics. The coefficients of seasonal and between-tree variations of total phenolics and of total LMWP were at a similar level through the growing season, but differences in individual LMWP were much larger. There was a clear difference between flavonoid and nonflavonoid compounds. The average coefficient of between-tree variation for flavonoids (except 32, 34, and 36) was 57%, whereas the average coefficient of seasonal variation was 22%; i.e., between-tree variation for flavonoid compounds was on average 2.6 times as large as temporal changes through the growing season. For nonflavonoid compounds the average coefficient of between-

tree variation varied between 65 and 72%, whereas the average coefficient of seasonal variation was 59%; i.e., between-tree variation for nonflavonoid compounds was less than half of the within-season variation.

Ecological Implications. The contents of total phenolics and of total LMWP decreased in the course of the season. Concentrations obviously had been even higher at the beginning of the summer, before our first sampling event, as indicated by high phenolic values in small, expanding leaves (unpublished). This contrasts with the common belief that foliage phenolics increase with season. The observation behind this opinion dates to Feeny (1970), who found that the content of condensed tannins in oak leaves increased from spring to autumn, and this observation provided a plausible explanation for the prevalence of spring feeding by insects. The mechanism by which these compounds distributed insect feeding was assumed to be binding of leaf proteins into indigestible complexes. At that time, tannins and phenolic compounds in general were assumed to be quantitative defenses, and nonspecific in action. However, with tannins and precursors of tannins, plants synthesize and accumulate in cells a great variety of LMWP: hydroxybenzoic and hydroxycinnamic acids, acetophenones, flavonoids, stilbenes, and lignans. The defensive functions of these compounds are not clear yet.

Although total phenolics in mountain birch foliage correlate negatively with performance of the most important insect herbivore of birch, the autumnal moth (Haukioja et al., 1985; Suomela et al., 1995), the main phenolic compound, chlorogenic acid, was not significant (Suomela et al., 1995). The biological relevance of this observation is unclear since the true role of chlorogenic acid and some other phenolic compounds may be expressed only after they have been oxidized by polyphenoloxidases or peroxidases (Appel, 1993) to quinones (Duffey et al., 1986). These highly reactive molecules undergo nucleophilic attack by the amino and sulfhydryl side chains of proteins, forming irreversible, covalent bonds (Hurrel et al., 1982; Pierpoint, 1983). The interaction of quinones with other components of cells (carbohydrates, free amino acids, and lipids) also is possible (Takechi and Tanaka, 1987; Felton et al., 1989).

A lack of the content of pooled phenolics on herbivores is further indicated by the fact that growth rates of larvae of a number of herbivorous species on mountain birch decreased strongly within the season (Hanhimäki et al., 1995). As both total phenolics and individual LMWP decreased within the season, the detrimental effects of phenolics cannot explain the reduced growth rate. As such, this is not surprising since the insensitivity of many herbivores to plant phenolics is well known (Bernays, 1981; Martin and Martin, 1982). They may not reduce digestibility (Martin et al., 1987), and the inhibition of protein digestibility may depend on the substrate (Duffey et al., 1986; Felton et al., 1989).

A potential source of confusion for the role of foliage phenolics for insects is that in many cases only soluble phenolics have been analyzed. At the present

time, however, it is well known that a number part of phenolic compounds (proanthocyanidins, some flavonoid glycosides, ferulic acid) are bound, apparently indissolubly, to plant tissue (Shen et al., 1986; Stafford, 1988; Strack et al., 1989). It has been suggested that these insoluble forms of plant polyphenols are bound by covalent linkages to polysaccharides or other structures in the developing cellular matrix. Attention has been drawn to the possible analogies with the structure of lignin and the process of lignification in forming xylem cells (Bate-Smith, 1954; Bate-Smith and Lerner, 1954; Shen et al., 1986; Haslam, 1988).

The mechanism of action of birch foliage phenolics on insects many depend on the specific chemical structure of the phenolics. Moreover, our results show that at different stages in birch leaf development, different groups of phenolic compounds may play a major role. A possible explanation for the decrease in the content of soluble phenolic compounds during the process of leaf maturation (see Table 1) may be transformation of soluble forms of phenolics to insoluble ones, for example, into the cell-wall bound compounds. Hence, the accumulation of phenolics in the cell wall of birch leaves and the increase of leaf toughness might be important causative factors in the decreasing suitability of birch leaves to some herbivores towards the end of growing season. Testing of this idea will require analysis of individual compounds and not of pooled contents of phenolics only.

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ANTIFOULING ACTIVITY OF *lyso*-PLATELET-ACTIVATING FACTOR EXTRACTED FROM AUSTRALIAN SPONGE *Crella incrustans*

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Abstract—Larvae of the ascidian *Clavelina moluccensis* do not settle in the field on the sponge *Crella incrustans* and are deterred from settling by crude chemical extracts of the sponge. Progressive refinement of extracts, guided by bioassays, led to the identification of *lyso*-platelet-activating factor (*lyso*-PAF) from the sponge. *lyso*-PAF is inhibitory in assays using ascidian, bryozoan, barnacle, and algal settlement and mussel byssal attachment. Its role in the field has not yet been demonstrated.

Key Words—*lyso*-Platelet-activating factor, *Crella incrustans*, *Clavelina moluccensis*, antifouling, fouling, marine settlement, marine chemical ecology, *Bugula*, *Xenostrobus*, *Balanus*, *Ulva*, mussel, sponge, ascidian.

INTRODUCTION

This paper originated from a research program on the ecology of coexisting, sessile, filter-feeding marine invertebrates on hard substrata (e.g., Butler, 1991; Butler and Chesson, 1990). The ecology of such organisms is characterized by intense competition at least for space, if not also for access to the water column with its suspended food (Buss, 1979a). These invertebrates also need defense against fouling by other organisms, predation by relatively large organisms, and

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infection by microorganisms. Our particular concern here is their capacity to resist macrofouling-settlement on them by other marine invertebrates.

Defenses may lie in structure (e.g., spines, skeletal inclusions) and in flexibility of growth form (Buss, 1979b; Buss and Jackson, 1981; Dyrynda, 1986; Jackson, 1977a,b), but it is clear that many have chemical defenses. These can protect them against settlement by larvae or overgrowth by adults of other species and, indeed, can enable them to gain further space for themselves or their progeny (Coll, 1992; Bakus et al., 1986, 1990; Davies et al., 1989; Dyrynda, 1986; Pawlik, 1992b; Richmond and Seed, 1991; Wahl, 1989; Holmström and Kjelleberg, 1994).

These defenses are of interest in themselves and for understanding the ecology of sessile assemblages. It is also reasonable to look among these organisms for potential natural antifouling agents that can be used to replace the effective but environmentally dangerous treatments based upon the heavy metals tin and copper (Claisse and Alzieu, 1993; Evans and Clarkson, 1993).

The mechanism of marine fouling on hard substrata involves an extremely complex interaction—not simply an obligate, linear succession—among (1) the adsorption of inert organic and inorganic molecules onto an underwater surface, (2) the development of a microbial film (microfouling), (3) the adsorption of inert particulate matter, and (4) the settlement and development of algal spores and invertebrate larvae (macrofouling) (Clare et al., 1992). There is little information about how these factors interact in the processes of organisms attaching to one another and hence little understanding of the various mechanisms by which they might resist it.

A first step is simple documentation of lack of fouling of certain sessile organisms (Davis et al., 1991; Dyrynda, 1986; Wahl and LaFargue, 1990; Walls et al., 1993); a second is the isolation and characterization of compounds that might be responsible for this activity. Further questions depend on the interest of the investigators. It may be asked whether a given molecule functions in the living organism in the way found with assays of pure compound. It is also important to know where the compound is located in the organism, how it is produced, and how its presence evolved (e.g., Coll, 1992; Maida et al., 1995; Schmitt et al., 1995; Walls et al., 1995). If the focus is on the discovery of new antifouling technology, then much of the foregoing may be of little interest and investigation may proceed directly to incorporation into coatings and testing in application (Price et al., 1992).

Davis et al. (1991) found that larvae of the ascidian, *Clavelina* (*Podoclavella*) *moluccensis* (Sluiter) (Clavelinidae: Aplousobranchia), strongly avoided settling on the sponge, *Crella incrustans* (Carter 1885) (Demospongiae: Poecilosclerida: Crellidae), among others. Indeed, *C. incrustans* in the field has almost no other macroorganisms growing on its surface. They also reported that crude chemical extracts of *C. incrustans* deterred larval settlement. This paper

shows that one pure compound isolated from *C. incrustans* has antifouling activity.

METHODS AND MATERIALS

Collection of Sponges

SCUBA divers collected sponge samples by hand from the pilings of the pier at Edithburgh, South Australia, at approximately 4–5 m depth. They were immediately stored at 0°C for transport and then kept frozen at −20°C until required. Since the isolation of compound **1** was bioassay driven and continued over an extended period of time, fresh collections of sponge were made prior to each bioassay period to guard against loss of activity by decomposition of the active components during storage. Sponges are notoriously difficult to identify underwater and therefore a voucher specimen of each sponge colony was lodged with the Queensland Museum (Brisbane, Australia). Its identity was confirmed as *Crella incrustans* (Carter, 1885) by Dr. John Hooper of the museum. Additional vouchers were lodged in the South Australian Museum, Adelaide, Australia.

Bioassays

The approach used was progressive refinement of sponge extracts, directed by bioassays, toward the isolation of single compounds. The chemical purification procedure began by partitioning crude acetone extract between ether and water, then between 1-butanol and water. The ether soluble compounds were then partitioned between hexanes and 20% aqueous methanol. The four extracts thus obtained were bioassayed, and the most active fraction was selected for the next step. The initial bioassays used the same species of colonial ascidian, *Clavelina moluccensis*, as that used by Davis et al. (1991), who made field observations on the deterrence of settlement of ascidian larvae by sponges and sponge extracts. Then, seeking wider evidence of activity, avoidance of species-specific artifacts, greater speed, sensitivity, and reduced costs, we moved to laboratory assays using bryozoan larvae, mussels, barnacle larvae and algal gametes.

Bioassay with Larvae of Clavelina moluccensis. Assays, conducted beneath the Edithburgh jetty, the site of collection of the sponge, were structured as follows. Extracts were dissolved in a solvent and painted onto one half of a 45-mm-diameter filter paper; the other side was painted with the solvent alone to act as control. Each filter paper was placed in the lid of a 70-ml screw-top polystyrene container, filled with seawater, taken underwater, and a single larva, caught in a syringe, was "injected" into each container. Thus, each larva provided a single, independent, binary choice between extract and control, which

occupied equal areas of paper symmetrically placed in the container. The containers were left underwater (cap and filter paper uppermost) for several hours to allow the larvae to settle, after which each container was scored as to whether settlement had occurred on the extract or control half of the filter paper. Other results were also observed, such as settlement on the walls of the plastic container or still swimming, but these were irrelevant for the purposes of statistical analysis. The null hypothesis for the assay was that if the extract had no influence, then, of those larvae that settled on the paper, half should be expected to settle on each side. The G test (log-likelihood ratio test) was used to test the fit to a 1:1 ratio and, where relevant, to test for differences between trials and between different extracts.

The test samples were applied to the Whatman No. 1 or Whatman No. 111 filter paper in the following manner. Filter papers were folded in half and the test material, generally dissolved either in diethyl ether or a 1:1 mixture of diethyl ether and methanol, was applied as a 0.1-ml aliquot over one half of the filter paper, taking care not to encroach onto the other half. The test sample half of the filter paper was distinguished by placing a small graphite pencil dot near the edge. Once the filter paper had dried, the same solvent (0.1 ml) used for the sample was applied to the other half of the filter paper to act as the control. In the case of compound **1** and related samples it was necessary to add chloroform to properly solubilize the sample. A typical solvent mixture was 11:10:7 methanol–chloroform–diethyl ether.

Bioassay with Larvae of Bugula neritina. The bryozoan *Bugula neritina* (Linnaeus) broods its developing larvae, and they are ready to settle shortly after release. Colonies of *B. neritina* collected from pontoons in a yacht basin at Outer Harbour, Port Adelaide, were kept in laboratory aquaria at 18°C in the dark for two to three days before being transferred to plastic food containers and exposed to bright light to induce larval release. Larvae were collected individually with a syringe and transferred with approximately 5 ml seawater to experimental trays.

The basic design was as for the *Clavelina* assay, a binary choice by a single larva in each container, leading to the same statistical test. The experimental containers were white square, high-impact polystyrene weigh trays, 34 mm interior length. A pencil line was drawn to divide the bottom into halves; one side was painted with extract and labeled E with pencil, the other painted with solvent. Generally several treatments (different extracts or different concentrations of the same extract) were run simultaneously. A control (solvent–solvent) comparison was included with each run. The control was painted with solvent on both sides, with one side being labeled C. This should conform to the 1:1 null hypothesis and should also give a high survival and settlement rate; if it did not, we would suspect that some other factor, e.g., condition of larvae or

seawater, was influencing results. Thirty or more trays were set up for each treatment. The solvent used was 3 : 1 ether–methanol.

Trays were positioned randomly to obviate possible effects of position on the bench. They were left for 4–14 hr, and larvae were scored as settled, not settled, or dead on either extract or solvent side. As with the *Clavelina* assay, the major statistical test was of the null hypothesis that of those larvae that did settle in choice trays, equal numbers would settle on the control and extract sides. A second G test compared the proportion dead in trays containing the extract (choice trays) with that in solvent–solvent controls. If significantly more were dead in choice trays, the extract was scored as "toxic."

Bioassay with Xenostrobus pulex. This method is modified after that used by Ina et al. (1989). Black mussels, *Xenostrobus pulex* (Lamarck), preferably about 1 cm in length, were collected from the breakwater at Gulf Point Marina, North Haven, South Australia. A small rubber stopper was fixed with Superglue to either the right or left valve of the shell, approximately 1 mm from the umbo on the ventral side, 30 on the right and 30 on the left valve per treatment. Four cards were set up (pasteboard, 7 × 13 cm) for each treatment. Ruled pencil lines divided the card into thirds parallel to the long axis. Extract was dissolved at known concentration into a known quantity (0.5 ml for four cards) of solvent (1 : 1 ether–methanol), which was painted evenly onto the central third of the card and on the two outer strips. On control cards solvent was applied to the whole card. Fifteen mussels were attached to each card by gluing their rubber stoppers on with Superglue. They were positioned along the pencil lines, eight on one side, seven the other, in an alternating pattern—umbos left–right–left, etc., to minimize the chance of a spurious result caused by any gradients in the room or by the asymmetry of mussels (Figure 1). Each mussel was positioned with its byssal gape above the pencil line; it could attach its byssal threads on either side of the line, and the null hypothesis was that if the extract had no influence, then 50% of them would be expected to attach towards the center of the card and 50% towards the outer side. Mussels would normally attach a number of byssal threads, some of which may be either side of the line, and each mussel was given a single score, viz., the side on which the greater number of threads was attached. Each card was placed in a separate food container, covered with seawater, left overnight at 18°C, then removed and scored. Results for the four cards of each treatment were tested for homogeneity and pooled if appropriate.

Bioassays Using Cyprid Settlement of Balanus amphitrite amphitrite *and Gamete Settlement and Germling Development of* Ulva lactuca. Both bioassays were performed by Dr. R. De Nys (School of Biological Sciences, University of New South Wales, Australia). The assay with cyprids of *Balanus amphitrite amphitrite* Darwin 1854 employed the method of Rittschof et al. (1992) as used

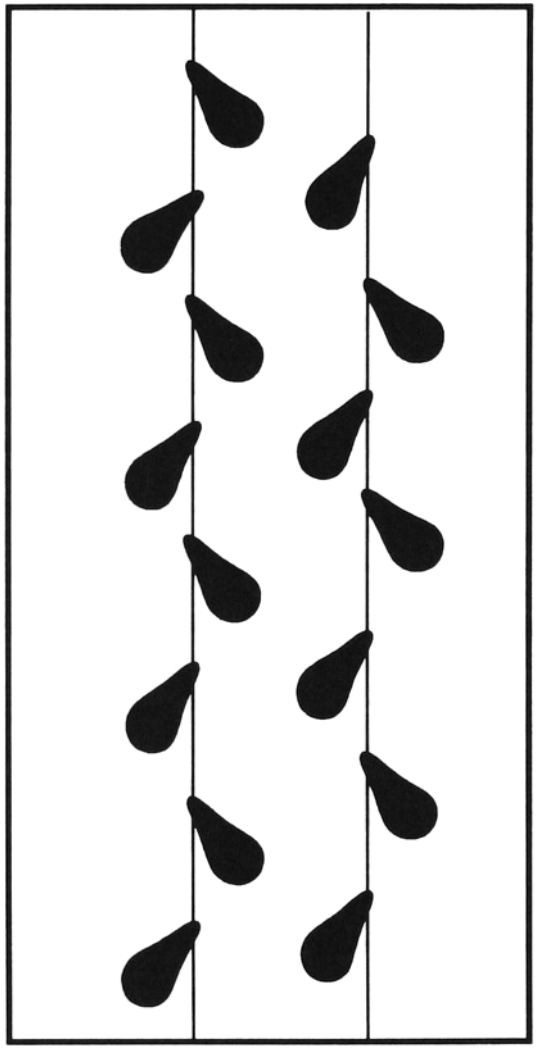

FIG. 1. Layout of mussels, *Xenostrobus pulex*, mounted on small rubber blocks above test card. Extract solution is painted between the lines, solvent control outside them.

by De Nys et al. (1995). Compound **1** was dissolved in ethanol (99.7% +) at concentrations ranging from 500 μg/ml to 50 ng/ml. A 1-ml aliquot of test solution was added to a polystyrene Petri dish (surface area 20 cm^2), resulting in a concentration range of 25 $\mu g/cm^2$ to 2.5 ng/cm^2. Control dishes were prepared, to which 1 ml of ethanol or no ethanol was added. Dishes were dried on a shaker prior to addition of 5 ml of sterile filtered (0.22 μm) seawater. Settlement assays were conducted by adding 45–65 cyprids to each dish. Each concentration of compound **1** was tested in triplicate. Test dishes were kept at 28°C in a 15L:9D cycle for 24 hr. After 24 hr, the test was terminated by addition of three drops of formaldehyde and nonsettled larvae filtered from the dish. Settled and nonsettled cyprids were counted.

Compound **1** was tested in a similar manner in the gamete bioassay with *Ulva lactuca* Linnaeus, in amounts ranging from 25 μg to 25 ng/cm^2 using the method from Fletcher (1989) modified by De Nys et al. (1995).

General Chemical Data

Infrared (IR) spectra were obtained on a Bio-Rad 3200 FT spectrophotometer. One dimensional ^1H, broad band decoupled ^{13}C, and DEPT NMR spectra were measured on either a Bruker ACP 300 MHz or AM 500 MHz spectrometers. Chemical shifts were assigned by comparison with shift of the carbon or residual proton in chloroform-d_1 (δ 77.0 and 7.24, respectively). Matrix-assisted laser-desorption ionization (MALDI) mass spectra were obtained with a Finnigan MAT Lasermat spectrometer in a 2,5-dihydroxybenzoic acid matrix.

All solvents were either HPLC grade or were redistilled from glass. Sephdadex LH-20 (Pharmacia) was used in partition and gel permeation chromatography with chloroform, hexanes, ethanol (10:10:1) and methanol, respectively, as the eluants. LiChroprep RP-18, 40–63 μm (Merck) reverse-phase silica gel was used for flash chromatography (Blunt et al., 1987). Normal-phase silica gel separations (Speedy column; Coll and Bowden, 1986), Merck Silica-gel H, and Chromatotron (Harrison Research; cornstarch incorporated with Merck Silica-gel 60 PF) were also attempted. Reverse-phase HPLC was carried out using a Waters isocratic system with a variable wavelength UV detector and an Exsil 100/10 ODS, 25 × 1.0-cm, semipreparative column (Activon). The course of separations was monitored by TLC (Merck, silica gel, 0.25-mm layer) using phosphomolybdic acid/ceric sulfate or Dragendorff's reagent for visualization.

Isolation of Compound 1

Typically, the frozen sponge (350 g, dry extracted weight) was chopped into 2-cm cubes and macerated in a Waring blendor with acetone, filtered through

diatomaceous earth, and then resuspended in fresh acetone. This procedure was repeated four times. Acetone was removed from the combined filtrate by rotary evaporation leaving an aqueous residue. The aqueous residue (500 ml) was extracted with 1-butanol (4 × 200 ml), dried under vacuum, and further partitioned between hexanes (400 ml) and 20% aqueous methanol (400 ml). The methanol layer was dried under vacuum to give 8.7 g sponge extract.

Purification and Identification of Compound 1

Crude extract (2.4 g) was partitioned on a Sephadex LH-20 column and the first fraction (0.72 g) was resubmitted to the column to give a fraction greatly enriched in compound **1** [R_f 0.2, 1-butanol, acetic acid, water (3 : 1 : 1); 0.28 g]). Final purification was achieved by reverse-phase flash chromatography (0.5% acetic acid in methanol) followed by reverse-phase HPLC (0.5% acetic acid in methanol). Fractions containing compound **1** were combined, and the solvent removed by rotatory evaporation to give 0.08 g of a white amorphous solid: mp 160°C (wide, decmpd); $[\alpha]_D$ − 3.5 (c = 0.63, $CH_3OH/CHCl_3$ 1 : 1); IR ($CHCl_3$ cast) 3200 (OH), 2915, 2859 (C–H), 1240, 1050, 960, 830, 750 cm^{-1}; ^1H NMR (500.1 MHz, $CDCl_3/CD_3OD$) δ 4.20 (bs, 2 H, $W_{1/2}$ 16 Hz), 3.90 (bt, 1 H, J = 10 Hz), 3.84 (m, 1 H), 3.77 (m, 1 H), 3.57 (bs, 2 H, $W_{1/2}$ 13 Hz), 3.36 (m, 6 H), 3.18 (s, 9 H), 1.47 (pentet, 2 H, J = 6.7 Hz), 1.18 (bs, 26 H), 0.80 (t, 3 H, J = 6.7 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3/CD_3OD$) δ 71.1 (s, CH_2), 70.0 (s, CH_2), 69.1 (d, CH, J = 7.3 Hz), 66.8 (d, CH_2, J = 5.0 Hz), 65.7 (bs, CH_2), 58.4 (bs, CH_2), 53.2 (bs, CH_3), 31.2 (s, CH_2), 29.0 (s, CH_2), 28.7 (s, CH_2), 25.4 (s, CH_2), 22.0 (s, CH_2), 13.1 (s, CH_3); MS (MALDI) m/z 482, Calc for $C_{24}H_{53}O_6PN$ (M + H): mol wt, 482.

RESULTS

Davis et al. (1991) had shown that crude extracts of *Crella incrustans* could deter the settlement of the larval ascidian *Clavelina moluccensis*. The major difficulty in isolating the active component in the crude material was the fact that the test organism was only available for a maximum of four weeks each year. Below we give a description of the isolation and identification of the settlement deterrent as it progressed over a number of years. The initial strategy, developed to make the most efficient use of each bioassay period, relied on testing fractions of the crude extract obtained by means of a variety of chromatographic methods just before the bioassay period with the hope of correlating the active fractions and being able to focus more quickly on the active component. As was discovered, this strategy had the added advantage of side-stepping problems of loss of activity due to the nature of a particular chromatographic procedure.

November 9–13, 1991

Crude acetone extract of sponge was partitioned between water and diethyl ether and then 1-butanol. The 1-butanol fraction was split in two, one half being submitted to gel permeation chromatography (giving fractions 3-161A–E) and the other chromatographed on silica-gel by Speedy column (giving fractions 3-161F–J). The diethyl ether portion was evaporated under vacuum and further partitioned between hexanes and 20% aqueous methanol. A portion of the methanol fraction was then partitioned on a Sephadex column to give seven fractions (3-159D–J).

Fractions 3-159F–I, 3-161A–C, and G, H, and J were tested against *C. moluccensis* larvae. Results for each fraction are given in Table 1, which includes results from some runs on the same extracts used as checks later in the season (November 22–27, below). The data for different runs on the same extract were not heterogeneous and have been pooled here.

Clearly, sample sizes achieved for some tests were small, the power of the tests is correspondingly low, and some nonsignificant results mean little, but 3-159G and H were significantly repellent and 3-161H significantly attractive (we use these terms to mean no more than the fact that more larvae settled away from or on the extract, respectively, without implying anything about the mechanism).

These results showed that: (1) the active component was not efficiently extracted into diethyl ether from the original acetone extract, (2) silica-gel chro-

TABLE 1. NOVEMBER 1991 *C. moluccencis* FIELD ASSAY RESULTS[a]

Fraction	Settled on ent side	Settled on extract side	Significance of difference from 1 : 1 ratio (P)	R/A
3-159F	6	8	NS	
3-159G	16	4	<0.05	R
3-159H	19	3	<0.01	R
3-159I	7	9	NS	
3-161A	13	5	NS	
3-161B	12	5	NS	
3-161C	3	4	NS	
3-161G	4	8	NS	
3-161H	1	6	<0.05	A
3-161J	5	8	NS	

[a]R = repellent; significantly more larvae settled on solvent side than on extract side; conversely, A = attractive, NS = $P > 0.05$. Significantly repellent fractions are labeled in **bold** type.

matography resulted in the loss of the active component, and (3) the active component was present in the middle fractions after partition chromatography and appeared to be in the early fractions after gel permeation chromatography.

November 22–27, 1991

The aqueous acetone extract from a new collection of *C. incrustans* was exhaustively extracted with diethyl ether, dried under vacuum, and partitioned between hexanes and 20% aqueous methanol. The methanol fraction was partitioned on Sephadex to give six fractions (3-185C–H), and the middle four (3-185D–G) were separated into nine fractions each (3-193, 3-187, 3-189, and 3-191A–I, respectively) by gel permeation chromatography.

Bioassays commenced with the larger mass fractions, which showed clearly defined spots by TLC. Initially a qualitative settling deterrent response was sought, and it was not until the fifth day of testing that a positive result was observed among early fractions from the gel permeation column (3-187A, 3-191A). On the sixth day, further testing of these fractions showed that the deterrent response was statistically significant. Activity appeared also to be present in 3-193A and 3-189B, although not statistically significantly; it was not possible to run further tests on these fractions. Bioassay data are summarized in Table 2, in which runs on a given extract have been pooled if not significantly heterogeneous. Again, some sample sizes are small and results should be treated cautiously, especially for those marked as having a low sample size, where cell expectations are three or less.

The ^{1}H and ^{13}C NMR of the chromatographically similar repellent fractions indicated that they consisted almost entirely of a single organic compound bearing glycerol and phosphocholine subunits reminiscent of a choline phospholipid. Visualization of TLCs of these fractions with Dragendorff's reagent confirmed the presence of a compound with a nitrogenous functional group.

These results clearly suggested the targeting of a particular compound in the sponge extract that could be detected by NMR, but partition chromatography appeared not to be an efficient means of purifying it.

November 4–8, 1993

An aqueous acetone extract from a new collection of *C. incrustans* was extracted with 1-butanol, dried under vacuum, and partitioned between hexanes and 20% aqueous methanol. The methanol fraction was submitted to two passes over gel permeation chromatography, and the fraction containing the bulk of the Dragendorff-positive component was separated by reverse-phase flash chromatography into seven fractions (4-127A–G). Fraction 4-127E was purified by reverse-phase HPLC to a single compound (4-129E) whose NMR spectra were essentially identical to those obtained for the previously active fractions. Frac-

TABLE 2. RESULTS FROM SECOND SERIES OF *C. moluccensis* FIELD ASSAYS, NOVEMBER 1991[a]

Fraction	Settled on solvent side	Settled on extract side	Significance of different from 1 : 1 ratio (P)	R/A
3-193A	5	1	NS	[b]
3-187A	11	3	<0.05	R
3-187B	2	1	NS	[b]
3-187C	3	9	NS	
3-187D	2	3	NS	[b]
3-187F	2	3	NS	[b]
3-187G	0	7	<0.01	A
3-187H	0	5	<0.01	A[b]
3-189A	5	7	NS	
3-189B	7	4	NS	
3-189C	3	3	NS	[b]
3-189D	4	7	NS	
3-189E	8	12	NS	
3-189F	10	8	NS	
3-189G	0	6	<0.01	A[b]
3-189H	10	3	<0.05	R
3-191A	18	5	<0.01	R
3-191B	5	4	NS	
3-191C	3	6	NS	
3-191D	4	6	NS	
3-191E	1	10	<0.05	A
3-191F	3	5	NS	
3-191G	6	6	NS	
3-191H	1	2	NS	[b]

[a]Conventions as in Table 1.
[b]Tests with low sample size.

tions 4-127B, C, and D, and the compound 4-129E were tested for settling deterrence against the larvae of *C. moluccenis*.

Several concentrations of 4-129E were compared in an attempt to determine an appropriate concentration for assays that would deviate significantly from a 1 : 1 ratio. Five concentrations were used (see Table 3) and the set of trials run over three days. The pooled data are given in Table 3. For 4-129E, the proportions settling on control versus extract papers were significantly heterogeneous between concentrations at the 5% level. Overall, the data deviated significantly at the 0.1% level from a 1 : 1 ratio: the fraction was clearly repellent. Although only the three highest concentrations were significant, the two lowest showed a trend in the same direction.

TABLE 3. TESTING OF FRACTIONS 4-127B, C, AND D, AND 4-129E WITH LARVAE OF *Clavelina moluccensis*, EDITHBURGH, NOVEMBER 1993[a]

Concentration ($\mu g/cm^2$)	Settled on paper (N)		Significance of deviation from 1:1 ratio (P)	R/A	Proportion settled on extract side
	Extract	Control			
4-127B					
12.46	10	14	NS		
4-127C					
16.73	7	11	NS		
4-127D					
12.58	6	23	<0.001	R	
4-129E					
12.77	5	33	<0.001	R	0.13
8.22	10	42	<0.001	R	0.19
3.84	11	23	<0.05	R	0.32
1.70	11	14	NS		0.44
0.86	9	13	NS		0.41

[a]Conventions as in Table 1.

Mussel and Bryozoan Bioassays

A sample of *C. incrustans* collected from Edithburgh in February 1992 was extracted with acetone and the resulting extract partitioned between hexanes, 20% aqueous methanol, 1-butanol, and water. The mussel assay was applied to all four fractions, with no significant effect detected. The hexanes and 1-butanol fractions were tested with *Bugula* at 130 ng/cm^2, both giving significant repellent effects. The effect of *lyso*-PAF should be seen in the 1-butanol and perhaps methanol fractions but definitely not in the hexanes fraction; the activity towards *Bugula* in those fractions must be due to some other compound(s).

From the November 13, 1991, collection of *C. incrustans* (which yielded the fractions 3-187, 3-189, 3-191, 3-193 used in the second series of field bioassays on *Clavelina*), pooled fractions 3-193B, 3-189B, and 3-191A were refractionated by a reverse-phase Sep-Pak Classic Cartridge C18 (Waters), methanol–water step gradient, to yield fractions 3-257A–D. Fractions 3-257A–C were tested against *Bugula*, and fractions C and D were pooled and further separated using a reverse-phase Sep-Pak, methanol–water step gradient, to yield six fractions, 3-259A–F, which were also tested against *Bugula*. The results revealed the following: Fraction 3-257A was toxic, 3-257B had no significant effect, 3-257C was toxic, 3-259A was repellent, 3-259B–E were all toxic, and 3-259F was repellent.

Clearly, there was something in these fractions that was strongly deterrent or toxic to *Bugula*. The activity detected with the *Clavelina* assay in the field was presumably present in these fractions and may have been responsible for the result; if so, it seems that *Bugula* is very sensitive to it. In retrospect, it would appear that a major proportion of this material was compound **1**, and these observations are consistent with our results on the pure compound.

Compound **1** (fraction 4-141B) was tested in the mussel and bryozoan assays, and a sample given to Dr. R. De Nys for the cyprid and *Ulva* assays. Results are summarized in Table 4.

In the mussel assay, only the highest concentration tested gave significant inhibition of byssal attachment. The bryozoan assay was not used at that concentration because the next two in the series were significantly toxic to the larvae; at 2.2 $\mu g/cm^2$ all larvae died, cf. 3% in control dishes; at 1.1 $\mu g/cm^2$, >50% died (cf. 15% in controls); at 546 ng/cm^2 and 273 ng/cm^2, death rates were not significantly different from controls but there was significant (5% level) avoidance of settlement on the extract. (At 546 ng/cm^2, however, there was significant heterogeneity between the three trials, one of them not showing deterrence). Below 273 ng/cm^2 neither toxic nor repellent effects were detected. In the *B. amphitrite* cyprid assay, compound **1** was inhibitory only at the highest concentration tested; at a surface concentration of 25 $\mu g/cm^2$, 23% of cyprids settled ($N = 3$, SE 3.35, each individual test involved ≥ 43 cyprids) compared

TABLE 4. RESULTS OF MUSSEL BRYOZOAN, CYPRID, AND *Ulva* ASSAYS WITH FRACTION 4-141B (Compound **1**)[a]

Concentration per unit area	Mussel	Bryozoan	Cyprid	*Ulva*
4.4 $\mu g/cm^{-2}$	R			
2.2 $\mu g/cm^{-2}$	NS	T		
1.1 $\mu g/cm^{-2}$	NS	T		
546 ng/cm^{-2}	NS	R		
273 ng/cm^{-2}		R		
136 ng/cm^{-2}		NS		
68 ng/cm^{-2}		NS		
25 $\mu g/cm^2$			R	R
2.5 $\mu g/cm^2$			NS	NS
50 ng/cm^2			NS	NS
25 ng/cm^2			NS	NS
2.5 ng/cm^2			NS	

[a]R = repellent; T = toxic.

to ca. 50% settlement in all other concentrations and ethanol and seawater controls, e.g., ethanol controls 47% ($N = 3$, SE 2.15). In the *Ulva* assay, there was zero settlement at 25 $\mu g/cm^2$, whereas at all lower concentrations, settlement was the same as controls, ca. 25 germlings/cm^2.

Identification of Compound 1

The most prominent signal in the ^{13}C NMR spectrum appeared at δ 29.0, which is typical of methylene groups in a long aliphatic chain. Signals at δ 13.1 (CH_3), 22.0 (CH_2), and 31.2 (CH_2) correspond to a methyl terminus at one end of the chain. The lack of a carbonyl signal in either the IR or ^{13}C NMR spectrum suggested that the chain was linked to the remainder of the molecule through an ether group rather than an ester. The carbons α, β, and γ to the ether linkage were detected at δ 70, 28.7, and 25.4, respectively. The remainder of the signals in the ^{13}C NMR spectrum are all grouped between δ 72 and 53, typical of carbons attached to heteroatoms. A notable feature of these signals was that almost all were either broadened or appeared as doublets, suggesting coupling to an atom like phosphorus. The ^1H spectrum, besides showing features consistent with a long aliphatic chain, had a 9H signal at δ 3.18, which suggested the presence of a trimethyl ammonium group in the structure and possibly a choline-based terminus. This is supported by the broad signal at δ 53.2 (CH_3), and further signals at δ 58.4 (CH_2) and 65.7 (CH_2) in the ^{13}C spectrum for the ethyl linkage between the two heteroatoms of choline. A third terminus was identified as an alcohol group from the IR spectrum (3200 cm^{-1}). On the basis of this analysis, it was suggested that the compound was a glycerophosphocholine with an unusual ether-linked aliphatic chain at one of the remaining alcoholic positions of glycerol, while the third remained unattached. Comparison of our NMR data with that of *lyso*-platelet activating factor (compound **1**) (Convert et al., 1984) showed that they were identical in all respects (Figure 2).

Two points remained to be determined about the structure, viz., the length of the alkyl chain and whether compound **1** was optically active. Since no sp^2 proton signals appeared in the ^1H spectrum, the alkyl chain length and the presence of homologs could be readily determined by low-resolution MALDI

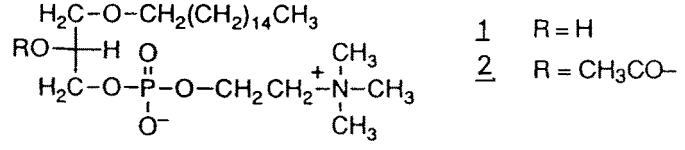

FIG. 2. Structure of compound **1**, *lyso*-PAF, and compound **2**, PAF.

mass spectrometry. In the event, only one significant peak was detected at *m/z* 482, $[M+H]^+$, which corresponds to a chain length of 16 carbons. The $[\alpha]_D$ value of $-3.5°$ indicated that compound **1** was not racemic and had the *R* or Sn configuration (Fusetani et al., 1986). Thus compound **1** was identified as 1-hexyldecyl-sn-glycerol-3-phosphorylcholine (*lyso*-PAF).

DISCUSSION

Sponges have been one of the groups of sessile invertebrates most actively studied by marine natural products chemists since, in an extremely competitive environment, they often appear to be undefended by any physical processes (Bakus et al., 1986; Davis et al., 1989) and they offer relatively large amounts of lipophilic material for investigation. Large numbers of compounds with novel structures and diverse biological activity have been found in sponges (Minale et al., 1976; Faulkner, 1994). Despite this level of activity, however, the role of these compounds in their environment has rarely been investigated. This is not surprising given the complexity of the task of testing hypotheses about ecologically significant chemical interactions in the marine environment.

Paul (1992) has noted three criteria that need to be addressed for the ecological significance of an antifouling compound to be established. They are: (1) Is the compound available to the potential fouling organism at sufficient concentrations to act as a deterrent? (2) Is the fouling species one that may potentially foul the organism in nature? (3) Does the compound deter fouling under natural conditions?

Jensen et al. (1990) have, in a different but relevant context, suggested two further criteria: (4) Is the compound isolated a true metabolite of the organism (or its symbionts) and not artificially derived during collection or extraction from an associated but otherwise inactive substrate? (5) Can a normally nondeterrent surface be made deterrent by application of the compound?

If antifouling activity is demonstrated in the field, we might go further and ask more detail about its origin—whether it is produced by the organism itself or by symbionts, and in either case by what biochemical pathway and with what evolutionary origin. These are interesting but not ecological questions; there is a temptation to move in their direction because they may be more tractable than the ecological questions, but the above ecological criteria must be met before these other questions have meaning.

In our procedure of identification of *lyso*-PAF as a fouling deterrent of the larvae of *C. moluccensis* we have largely satisfied four of the five criteria. *C. incrustans* and *C. moluccensis* share a common environment, and it has been shown that the surface of *C. incrustans* is significantly avoided by the larval ascidian (Davis et al., 1991). The bioassays were of necessity conducted under

natural conditions to the extent that wild larvae were used as the test organisms and with regard to seawater constitution and temperature. The test used in the bioassay was a test of the difference between surfaces (viz., filter paper) coated and not coated with the compound. *C. incrustans*, when collected, was noticeably free of epibionts and readily torn off from above its substratum (a timber piling), thus giving confidence that the source of *lyso*-PAF is the sponge itself. The reproducibility of the extraction of the antifouling component in the sponge and the inherent stability of *lyso*-PAF points to it being a true metabolite of *C. incrustans* (or of closely associated commensals). Paul's (1992) first question concerning the available concentration of *lyso*-PAF encountered by the settling *C. moluccensis* larvae remains to be answered; we cannot say whether it is present at the surface of the undisturbed sponge and hence able to influence a larva on approach or contact. Nevertheless, we have clearly shown it is active as an antifouling agent and therefore it was interesting to ascertain its activity against a variety of fouling organisms. Besides the original *C. moluccensis* larvae, *lyso*-PAF was tested for settlement deterrence in four other assays. Some of these have been used in other laboratories and, as a result, it is possible to compare the activity of *lyso*-PAF with that of other compounds known to have antifouling properties.

In the *Balanus amphitrite* cyprid assay, 25 $\mu g/cm^2$ of *lyso*-PAF yielded significant inhibition (23% settlement vs. 47% in ethanol controls); compare the *Delisea pulchra* extract, 0% settlement at 2.5 $\mu g/cm^2$ (De Nys, personal communication) and the near zero value for bacterium D2 films $\leq$48 hr old (Holmström et al., 1992). Kon-ya et al. (1994) and Keifer et al. (1986) report relevant results for 2,5,6-tribromo-1-methylgramine (TBG) and renillafoulins, but it is difficult to compare these concentrations in solution with our concentrations per unit area.

In the *Bugula neritina* larval assay, *lyso*-PAF was toxic above 1.1 $\mu g/cm^2$ and gave significant settlement inhibition above 273 ng/cm^2, whereas renillafoulins did not deter larvae of *B. neritina* (Davis et al., 1989; Pawlik, 1992b). When *lyso*-PAF was tested in the assay using gametes of *Ulva lactuca*, there was zero settlement at 25 $\mu g/cm^2$ but no inhibition at lower concentrations; compare the *Delisea pulchra* extract, which yielded zero settlement at 25 ng/cm^2 (De Nys, personal communication).

Byssal thread attachment by *Xenostrobus pulex* was significantly inhibited at 4.37 $\mu g/cm^2$. In a similar assay using *Mytilus edulis*, 80 $\mu g/cm^2$ $CuSO_4$ was the concentration required for maximal repellant activity in a five-step scoring system starting at zero activity (Ina et al., 1989), while the comparable value for crude MeOH extracts from *Undaria pinnatifida* and *Costaria costata* was 4 mg/cm^2, and for purified glycerolipids from them, on the order of 1 $\mu mol/cm^2$ (Katsuoka et al., 1990). Clearly, the results are mixed, and it is difficult to

compare the activity of *lyso*-PAF with that of other fouling deterrents, but the assays all indicate a deterrent effect.

In testing compound **1** itself (Table 4), the bryozoan assay was much more sensitive than the mussel, cyprid, and *Ulva* assays. The mussel assay detected repellent activity only at 4.4 $\mu g/cm^2$, while the bryozoan assay showed toxicity down to 1.1 $\mu g/cm^2$ and repellence down to 273 ng/cm^2. The cyprid and *Ulva* assays showed repellence at 25 $\mu g/cm^2$. The *Clavelina* assay detected repellence at and above 3.8 $\mu g/cm^2$. This difference in sensitivity of the assays appears to be general. Apart from *Crella incrustans*, in our program, so far 17 species (14 sponges, 3 ascidians) have been tested using various extraction procedures, and the *Bugula* assay has detected repellent activity in more fractions than *Xenostrobus*. Most of the active fractions have been polar lipophilic compounds (soluble in "wet" ether) with molecular weights between approximately 600 and 1500 amu as estimated from their behavior in gel permeation chromatography (i.e., Sephadex LH-20 with methanol or methanol–chloroform). *B. neritina* responded to at least one fraction from every species tested. Despite their cosmopolitan distribution, *B. neritina* larvae seem to be deterred from settling by a wide variety of marine organism extracts; for this reason we consider them of limited value in antifouling assays.

Compound **1**, *lyso*-PAF, besides being present in a variety of mammals including humans, has been found in a hydroid (Fusetani et al., 1986), a starfish (Iorizzi et al., 1991), and an earthworm (Noda et al., 1992). The identification of *lyso*-PAF as a settling deterrent raises several questions. How does its chemical structure relate to its deterrent activity, how does it compare with the structures of other putative settling deterrents, and does the vast amount of knowledge available about its action in mammalian systems suggest any insight into its action as a settling deterrent of marine invertebrates?

We shall not detail the relevant arguments and literature here, but outline the possibilities briefly. Antifouling activity has been attributed to certain nitrogen heterocycles (Targett et al., 1983; Kon-ya et al., 1994; Walls et al., 1995), and this activity may be due to a cationic group that may interfere with ion transport mechanisms; *lyso*-PAF bears such a cationic center due to a quaternary ammonium group. Another group of deterrent compounds can be said to have a broadly detergent-like structure, with a polar head group and a nonpolar tail (e.g., Mizobuchi et al., 1993), and we note that *lyso*-PAF has the overall structure of a detergent, with the trimethyl ammonium as the polar headgroup and a long aliphatic ether as the hydrophobic tail. It has been shown to be cytolytic and membrane perturbing (Weltzien, 1979). This possibility may be testable, because *lyso*-PAF is optically active. A significant reduction of deterrent activity of the unnatural *S* enantiomer compared to the *R* enantiomer would imply the existence of a specific receptor site on the *C. moluccensis* larva, rather

than some general detergent-like activity. We have recently initiated a structure–function study that seeks to determine the importance of the aliphatic chain length, the presence of the chiral center, and the ammonium group to the antifouling activity of *lyso*-PAF.

Almost nothing is known about the biochemistry of settling deterrence in marine invertebrates, but there are some remarkable similarities in the biochemistries of settlement induction and of PAF (Morse, 1990; Pawlik, 1988, 1990, 1992a,b), and one might pursue these on the assumption that the deterrence and induction of settlement are linked biochemical pathways. The most interesting similarities are in the roles of inorganic cations and of arachidonic acid as they relate to signal transduction mechanisms.

This paper concentrates on antifouling activity, which was the genesis of the work, but of course sessile invertebrates such as *C. incrustans* are exposed to other risks (predation, infection), and there are many mechanisms that may function to defend the organisms from these risks. Further, any of these mechanisms may have more than one function (Paul, 1992; Schmitt et al., 1995). We make no attempt here to examine other possible functions or to postulate evolutionary or immediate biochemical origins. These are important topics for further work.

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SEX RECOGNITION IN *Diglyphus isaea* WALKER (HYMENOPTERA: EULOPHIDAE): ROLE OF AN UNCOMMON FAMILY OF BEHAVIORALLY ACTIVE COMPOUNDS

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Abstract—The sexual behavior of the ectoparasitoid *Diglyphus isaea* is described. Recognition of the female by the male occurs at close range. Males initiate courtship behavior in the presence of a living female regardless of age, as well as in the presence of a female killed by freezing. Courtship behavior is not observed in the presence of a dead female washed with organic solvents but could be elicited using a lure covered with a female organic extract. These findings demonstrate that each sex develops a specific chemical signature that can be dissolved in hexane and transferred to a lure. Analysis of organic extracts by gas chromatography revealed chemical dimorphism between the two sexes. Gas chromatography coupled with mass spectrometry showed that the main components in females were esters of medium-chain fatty acids and long-chain 11-alcohols. There were few hydrocarbons. Female esters, which were present in only small proportions in males, were recovered in the nonhydrocarbon fraction obtained after fractionation of the total extract on a silica-filled microcolumn as a mixture containing 11-heneicosyl, 11-docosyl, 11-tricosyl, 11-tetracosyl, and 11-pentacosyl octanoate, and 11-docosyl, 11-tricosyl, 11-tetracosyl, and 11-pentacosyl decanoate. These results demonstrate that there is a specific gender-related chemical signature.

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Key Words—Hymenoptera, Eulophidae, *Diglyphus isaea*, courtship behavior, contact pheromones.

INTRODUCTION

The entomophagous hymenopteran *Diglyphus isaea* Walker is a larval ectoparasitoid that attacks numerous leafminer species including *Liriomyza trifolii* Burgess. The mating behavior of *Diglyphus* has not been previously studied, but we suspected that, as in many insects, pheromones allow sex recognition and possibly induce the courtship dance.

An obvious prerequisite for mating is the male and female encounter. Several factors complicate encounters in *Diglyphus*. First, unlike other parasitoid Hymenoptera, e.g., *Trichograma* (Suzuki and Hiehatri, 1985), in which encounters are promoted by infestation of the same host, *Diglyphus* is a nongregarious parasitoid and the likelihood that a male and female should emerge in the same place is low. Second, protandrism is well documented in parasitoid Hymenoptera (Suzuki and Hiehatri, 1985). Third, male *Diglyphus* develop more quickly (Minkenberg, 1989; Cheah, 1987) and thus emerge before females.

Our preliminary studies ruled out emission of volatile pheromones by either sex and showed that sex encounters take place on leaves parasitized by leafminer larvae to which both sexes are attracted by plant volatiles (Finidori-Logli, 1994; Finidori-Logli et al., 1996). This finding accounted for how the two sexes came together in the same place and time, but did not explain of how male and female *Diglyphus isaea* recognize one another on the same leaf.

In the present study, ethological tests using the chemical lure method (Howard et al., 1982; Bonavita-Cougourdan et al., 1987; Bagnères et al., 1991) were performed to assess the role played by chemical signals in the strategy used by males and females to recognize one another for mating. Contact sex pheromones have been documented in several invertebrate groups, e.g., Diptera (Uebel et al., 1977; Carlson et al., 1978; Huyton et al., 1980; Antony and Jallon, 1982), Orthoptera (Warthen et al., 1981), and spiders (Hegdekar and Dondale, 1969).

METHODS AND MATERIALS

Insects. Diglyphus used in these experiments were reared according to the "alternate open-field and caged ovipositing" method (Lyon, 1976) at a permanent rearing facility set up by the INRA in Antibes, France.

Ethological Testing. Experiments consisted of comparing the attractive effects of a living female and five different lures on males. The first lure was a female killed by freezing. The second lure was a female killed by freezing and

washed twice for 10 min in hexane. After the second washing, gas chromatography (GC) was performed on an extract to ensure that no more chemical signals were present on the cuticule. The third lure was a female killed by freezing, washed in hexane, and then covered with the whole extract obtained as described below. The fourth lure was a female killed by freezing, washed in hexane, and then covered with the apolar fraction of the whole extract. The fifth lure was a female killed by freezing, washed in hexane, and then covered with the polar fraction of the whole extract.

Males were tested individually in the presence of one female, or one of the five lures. All tests were carried out on the bottom of a Petri dish (6 cm diameter) covered with damp filter paper. Each test was replicated 20 times. The following five responses characteristic of courtship behavior were quantified: lateral trembling (I), mounting the female (II), palpating with antennae (III), genital contact (IV), and copulation (V). Statistical comparison of the male responses in the different test series was done using the Mann-Whitney test in a Statgraphics software (version 3.0).

Chemical Analysis. Extracts used to prepare lures for ethological tests were obtained from 200 females in 400 μl of hexane. These extracts were applied either whole or separately as apolar and polar fractions. Separation was performed on a microcolumn constructed by packing a capillary tube (8 cm length, 0.5 cm diameter) with Chromagel silica (particle size: 40–60 μm, 230–400 meshs) that was rinsed and dried in a drying chamber. The apolar fraction was obtained by elution with hexane. The polar fraction was obtained by elution with a mixture of hexane and diethylether (95:5).

For GC analysis, 20 extracts of each sex were obtained by immersion in hexane and concentrated under nitrogen. Females and males were killed by freezing and weighed. Female extracts were obtained from 20 individuals and male extracts from 40 individuals. GC was performed on a Delsi-Nermag system DN 200 equipped with a flame-ionization detector (FID), a split/splitless injector (splitss 15 sec), and a Chrompack CPSil5 CB apolar capillary column (25 m $\times$ 0.25 mm $\times$ 0.25 μm). The carrier gas was helium at 1 bar. Temperature was programmed from 70°C to 150°C at 30°C/min, and then from 150°C to 320°C at 5°C/min. Compounds were quantified using an *n*-alkane not present in the natural extract as internal standard. For females, 50 μl of a solution containing 400 ng of heneicosane (n-C_{21}) was added to the total extract. For males the same amount of nonacosane (n-C_{19}) was added. Equivalent chain length (ECL) (Miwa, 1963) was calculated for each compound using a mixture of internal standards from the n-C_{18} to the n-C_{40}, coinjected with the total extract.

Results were collected on an Enica 21 integrator. Quantifications were performed using a Lotus 1.2.3 spreadsheet (version 3.1) after correction of peak surfaces by applying the coefficient of Bagnères et al. (1991), to take into

account the sensitivity of the FID. Each product was quantified as a percentage (relative proportion), quantity per insect, and quantity per milligram of insect body weight.

For identification of female and male extracts, analyses were performed by electron impact (at 70 eV) followed by examination under positive chemical ionization ($CI^+/CH_4{}^+$, 150 eV) on a Hewlett-Packard HP 5890 Series II chromatography system coupled to a quadrupole MS Engine Hewlett-Packard 5989A mass spectrometer. The coupled system was controlled by a Chemsystem HPUX management system running Unix software. Carrier gas pressure (helium) was 2 bars with a source temperature of 240°C, a quadrupole temperature of 100°C, and an interface of 300°C. Masses were scanned from 45 (95 in CI^+) to 600 *m/z* at a mean rate of one scan per second. The chromatography system was equipped with an HP5 apolar capillary column (30 m × 0.25 mm × 0.25 μm). In addition, a GC-MS system VG 70/250 SE connected to an HP 5890 gas chromatograph, was used at 70 eV under the conditions described earlier (Schmitt et al., 1991).

Identification of Behaviorally Active Compounds. Identification of the natural aphrodisiac compounds was achieved by comparison with the following synthetic compounds, not commercially available.

Esters of Secondary Alcohols. Synthesis of reference compounds followed standard procedures (Weygand and Hilgetag, 1970). The 11-alkanols were prepared from undecanal (Merck) and appropriate Grignard reagents obtained from alkylbromides (Merck) in diethylether. Esterification was performed by reaction of the alcohol and corresponding acylhalogenide in pyridine.

Derivatization Reaction. Reduction of naturally occurring esters was carried out as follows: the polar fraction obtained from 400 females was brought to a volume of 100 μl. To this solution, 20 mg of lithium alumina hydride (carefully prewashed with pentane) was added. After 30 min a few drops of water were added and the organic layer was separated. After flash chromatography on Florisil (hexane–diethylether 95:5) and concentration, the sample was submitted to GC-MS (VG instrument) analysis without further purification.

RESULTS

Description of Courtship Behavior. After introduction into the Petri dish, males and females first explore the new surroundings. This exploratory phase is characterized by walking and leaping on the bottom, walls, and cover of the dish. The antennae are in constant movement. In all tests males recognized females only at close range (a few millimeters). Regardless of the age of the female, the male initiated the following courtship dance. First the male stops near the female and begins lateral oscillations with its wings extended towards

the female (20 of 20 tests). Next the male mounts on the back of the female and begins palpating her with his antennae. A receptive female allows this palpation while an unreceptive one flees. The male usually gives up after two unsuccessful attempts. If consent is obtained, the genitals are brought into contact as palpation with the antennae continues, and finally copulation with insemination occurs. There is no identifiable postcopulation behavior.

Courtship behavior using females killed by freezing was initiated in 19 of 20 tests (Table 1, Figure 1). Palpation with the antennae occurred in only 10/20 tests, genital contact in only 7/20, and copulation in only 2/20 (i.e., 10% success rate). Failure to complete courtship dance, especially in the last three responses, was probably due to the immobility of the female, which may cause the male to lose interest. Thus, while chemical signals are certainly involved in triggering courtship, these observations suggest that additional signals are necessary for completion of courtship.

Courtship behavior was not observed in any of the 20 tests carried out using a female that had been washed in hexane (Table 1, Figure 1). This finding confirms the aforesaid hypothesis that recognition of the female by the male

TABLE 1. MALE BEHAVIOR IN PRESENCE OF LIVING FEMALE (L), DEAD FEMALE (D), FEMALE WASHED WITH HEXANE (W), OR LURE COVERED WITH TOTAL EXTRACT (W+T), POLAR EXTRACT (W+P) OR APOLAR EXTRACT (W+A)[a]

Females or lures	Male behavior				
	I	II	III	IV	V
L	20	19	17	17	15
D	19	14	10	7	2
W	0	0	0	0	0
W + T	20	14	11	3	3
W + P	20	18	13	10	8
W + A	8	6	2	1	0

[a]The behavioral items observed were (I) lateral trembling, (II) mounting on the female, (III) palpating with the antenna, (IV) genital contact, and (V) copulation. Male responses were compared using the Mann-Whitney test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

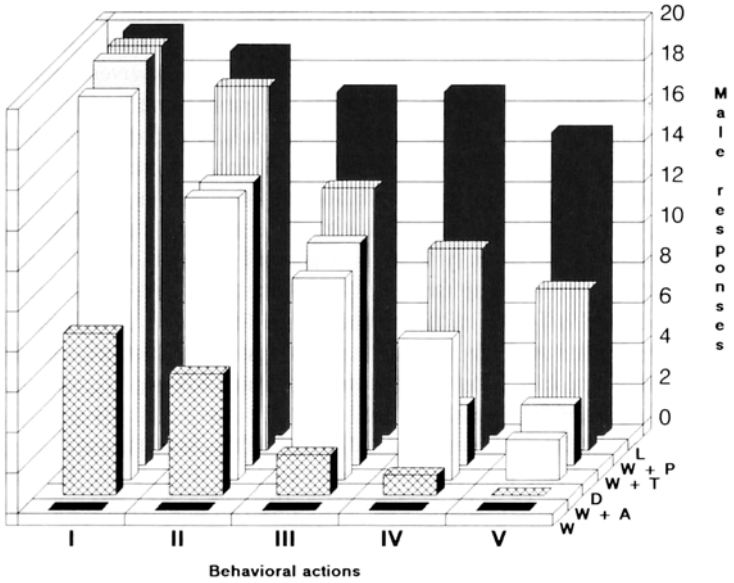

FIG. 1. Results of lure tests. The x axis indicates five items of courtship behavior: (I) lateral trembling, (II) mounting, (III) palpating with the antenna, (IV) genital contact, (V) copulation. The y axis indicates the number of positive male responses, N (number of males) = 20. The z axis indicates the test situation: W, male in the presence of a female washed with hexane (i.e., lure); W + A, male in the presence of a lure covered with apolar extract; D, male in the presence of a dead female; W + T, male in the presence of a lure covered with total extract, W + P, male in the presence of a lure covered with polar extract, L, male in the presence of a living female.

depends on cuticular chemical signals. A few preliminary bioassays were performed using washed lures covered with male extracts. No male response was ever observed, thus demonstrating that the aphrodisiac signal came exclusively from the female.

Courtship behavior resumed when a lure covered with 2 μl of the total hexane extract (i.e., the equivalent of one female) was used. Lateral oscillations were observed in 20 of 20 tests and mounting in 14 of 20 (Table 1, Figure 1). This finding supports the assumption that the chemical signal is located on the cuticle.

Lures prepared using the apolar phase did not restore courtship behavior in males. Lateral oscillations were observed in only 8 of 20 tests and copulation was never observed (Table 1, Figure 1).

In contrast, lures prepared with the polar phase restored courtship behavior in males. The first four responses of the courtship dance were observed in all 20 tests. Copulation was observed in 8 of 20 tests. This demonstrates that the polar fraction carries the most active principle for recognition of a sex partner.

Chemical Analysis. GC-MS analyses revealed qualitative and quantitative gender-related differences (sexual dimorphism) in the cuticular composition of *Diglyphus isaea* (Figure 2). Analysis of total extracts from males and females showed that most prominent products were apolar or moderately polar molecules, with long chains with 16–33 carbon atoms. These are classic findings for cuticular lipid extracts (Lockey, 1988; de Renobales et al., 1991). These substances can be divided into two groups, i.e., hydrocarbons and esters. Compounds identified in the cuticle of *D. isaea* (males and females), and corresponding amounts quantified in the stable part of the extract (from n-C_{23}) are listed in Table 2. Eleven common acids, methyl esters, or ethyl esters with C_{16} and C_{18} were detected only sporadically and are not included in the table.

In extracts obtained from males (mean weight $\cong$ 0.04 mg), the main components were hydrocarbons, (more than 80% of the cuticular mixture, see Table 2). These hydrocarbons are all saturated and can be classified into two categories: n-alkanes, which account for 16% of the cuticular extract (0.7 μg/mg

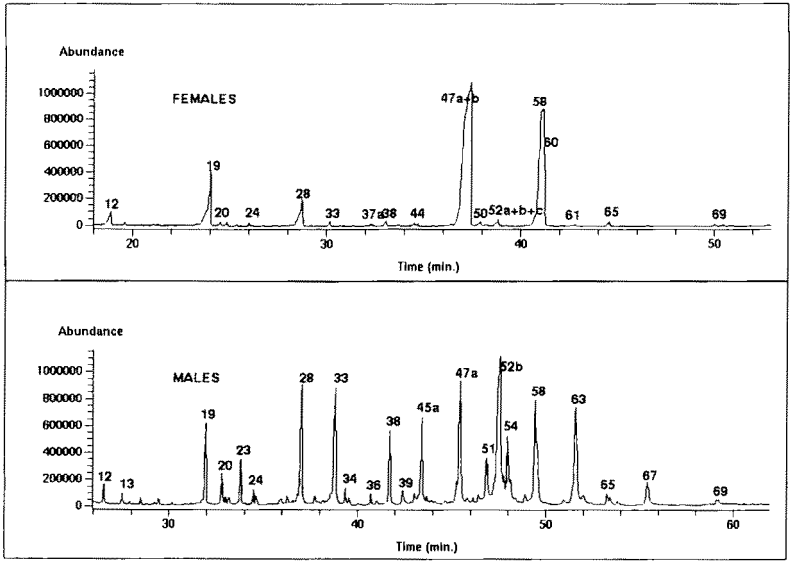

FIG. 2. Total ion chromatograms (TIC) of female (top) and male (bottom) *Diglyphus isaea*. For peak numbers see Table 2.

TABLE 2. COMPOSITION AND GC-MS IDENTIFICATION OF MALE AND FEMALE *Diglyphus isaea* EXTRACTS[a]

Peaks	Compounds	Males		Females		Carbons			Diagnostic ions
		Percentages	Quantity (ng/mg ins.)	Percentages	Quantity (ng/mg ins.)	ECL	(*N*)	MS/EI	$Cl(CH_4^+)$
12	*n*-Tricosane	0.38 (0.45)	23.81 (40.2)	1.13 (0.45)	46.18 (16.71)	23.00	23	324	323
13	11-Methyltricosane	0.13 (0.3)	3.1 (5.65)	0.08 (0.34)	2.58 (11.25)	23.35	24	168/9, 196/7	337
14	5-Methyltricosane	0.04 (0.07)	1.77 (4.27)			23.37	24	84/5, 280/1	337
15	3-Methyltricosane	0.08 (0.13)	2.83 (3.28)	traces	traces	23.67	24	56/7, 308/9	337
16	*n*-Tetracosane	0.07 (0.04)	2.93 (2.43)	0.005 (0.02)	0.23 (1)	24.00	24	338	337
17	4,10-Dimethyltricosane	0.09 (0.11)	3.38 (3.52)			24.09	25	70/1, 168/9, 210/1, 308/9	351
18	Unknown	1.20 (0.84)	traces			24.87			
19	*n*-Pentacosane	2.50 (1.65)	129.4 (159.22)	6.12 (0.68)	241.71 (74.04)	25.00	25	352	351
20	11-+13-Methylpentacosane	0.32 (0.72)	10.81 (14.97)	0.02 (0.05)	0.82 (2.76)	25.31	26	168/9, 196/7, 224/5	365
21	7-Methylpentacosane	0.10 (1.1)	5.36 (6.47)			25.40	26	112/3, 280/1	365
22	5-Methylpentacosane	0.05 (0.07)	2.38 (3.13)			25.50	26	84/5, 308/9	365
23	3-Methylpentacosane	0.93 (0.45)	41.21 (36.27)	0.01 (0.02)	0.28 (1.2)	25.68	26	56/7, 336/7	365
24	*n*-Hexacosane	0.37 (0.14)	16.37 (13.01)	0.33 (0.13)	13.43 (5.1)	26.00	26	366	365
25	4,12-Dimethylpentacosane	0.12 (0.13)	4.97 (5.54)			26.06	26	70/1, 196/7, 210/1, 336/7	379
26	4-Methylhexacosane	0.30 (0.24)	13.51 (12.07)			26.25	27	70/1, 336/7	379
27	3-Methylhexacosane	0.25 (0.1)	12.14 (10.34)			26.68	27	56/7, 350/1	379
28	*n*-Heptacosane	5.51 (1.82)	238.58 (175.99)	6.82 (1.12)	280.82 (56.41)	27.00	27	380	379
29	11-+13-Methylheptacosane	1.17 (1.13)	6.92 (5.62)			27.28	27	168/9, 196/7, 224/5, 252/3	393
30	7-Methylheptacosane	0.08 (0.03)	3.43 (3.43)			27.35	27	112/3, 308/9	393
31	5-Methylheptacosane	0.08 (0.04)	3.69 (3.71)			27.50	28	84/5, 336/7	393
32	11,15-Dimethylheptacosane	0.13 (0.07)	6.16 (5.62)			27.70	29	168/9, 196/7, 238/9, 266/7	407

33	3-Methylheptacosane	6.73 (2.53)	295.29 (238.72)	0.84 (0.38)	36.43 (20.49)	27.71	28	56/7, 364/5	393
34	*n*-Octacosane	0.62 (0.26)	25.29 (18.48)	0.15 (0.13)	6.22 (5.52)	28.00	28	394	394
35	4,18-+4,20-Dimethylheptacosane	0.21 (0.06)	9.33 (7.2)			28.07	29	70/1, 126/7, 154/5, 280/1, 308/9, 365/6	407
36	4-Methyloctacosne	0.48 (0.1)	21.48 (16.8)			28.57	29	70/1, 365/6	407
37a	11-Heneicosyl octanoate	0.19 (0.16)	8.62 (7.3)	0.26 (0.15)	10.35 (6.58)	28.71	29	127, 145, 294/5	437
37b	3-Methyloctacosane					28.71	29	56/7,350/1	407
38	*n*-Nonacosane	3.33 (2.95)	125.23 (98.47)	1.19 (0.25)	49.42 (14.33)	29.00	29	408	407
39	11-+13-+15-Methylnonacosane	1.49 (2.22)	62.41 (99.19)	0.02 (0.06)	0.96 (2.9)	29.28	30	168/9, 196/7, 252/3, 280/1	421
40	9-Methylnonacosane	traces	traces			28.38	30	140/1, 308/9	421
41	7-Methylnonacosane	0.13 (0.18)	5.29 (5.92)			29.42	30	112/3, 336/7	421
42	5-Methylnonacosane	0.11 (0.05)	4.78 (3.82)			29.50	30	84/5, 364/5	421
43	11,15-+11,17-Dimethylnonacosane +					29.60	30	168/9, 196/7, 224/5 238/9, 294/5	435
44	11-Docosyl octanoate	0.63 (0.2)	27.94 (21.30)	0.54 (0.14)	22.8 (8.27)	29.70	30	127, 145, 308/9	451
45a	3-Methylnonacosane	3.73 (1.48)	145.15 (103.91)	0.84 (0.38)	36.65 (20.93)	29.71	30	56/7, 392/3	421
45b	Trimethylnonacosane	traces	traces			29.71	32		449
45c	Trimethylnonacosane	traces	traces			29.71	32		449
46a	*n*-Triacontane	2.50 (0.16)	131.02 (139.79)	0.7 (0.34)	29.48 (14.6)	30.00	30	422	421
46b	Dimethylnonacosane	traces	traces			30.01	31		435
47a	11-Tricosyl octanoate	7.70 (3.45)	405.58 (429.58)	30.66 (1.92)	1288.56 (317.63)	30.63	31	127, 145, 322/3	465
47b	4-Methyltriacontane	traces	traces			30.64	31	70/1, 392/3	435
48	Dimethyltriacontane	4.93 (4.87)	282.55 (476.36)			30.72	32		449
49	*n*-Hentriacontane	0.26 (0.13)	13.77 (20.26)			31.00	31	436	435
50	Cholesterol	0.31 (0.17)	12.59 (8.93)	0.65 (0.46)	25.2 (20.33)	31.01	27	386	385
51	15-+13-+11-Methylhentriacontane	2.16 (0.76)	90.16 (63.24)	0.58 (0.63)	25.2 (30.26)	31.44	32	168/9, 196/7, 224/5 252/3, 280/1, 308/9	449
52a	11-Docosyl decanoate	traces	traces	traces	traces	31.45		155, 173, 308	479
52b	11,15-Dimethylhentriacontane	17.08 (4.64)	705.18 (444.06)	6.12 (2.73)	264.68 (137.78)	31.46	32	168/9, 238/9, 252/3 322/3	463
52c	11-Tetracosyl octanoate			0.45 (0.31)	17.98 (13.86)	31.50	32	127, 145, 336/7	479
53	3-Methylhentriacontane	0.75 (0.28)	28.14 (17.31)			31.66	32	56/7, 420/1	449

TABLE 2. CONTINUED

Peaks	Compounds	Males		Females		Carbons		MS/EI	Diagnostic ions $Cl(CH_4^+)$
		Percentages	Quantity (ng/mg ins.)	Percentages	Quantity (ng/mg ins.)	ECL	(N)		
54	11,15,19-Trimethylhentriacontane	3.15 (0.9)	135.07 (99.51)			31.73	34	168/9, 196/7, 238/9 266/7, 308/9, 336/7	477
55	7,11,15-Trimethylhentriacontane	0.72 (0.23)	30.80 (22.13)			31.86	34	112/3, 182/3, 252/3, 322/3, 392/3	477
56	Dimethylhentriacontane	0.35 (0.22)	14.25 (9.96)			32.08	33		463
57	Trimethylhentriacontane	0.28 (0.27)	11.84 (10.71)			32.16	34		477
58	11-Tricosyl decanoate	4.17 (4.13)	240.46 (391.75)			32.33	33	155, 173, 322/3	493
59	Dimethyldocotriacontane	0.95 (0.72)	37.98 (35.47)			32.41	34		477
60	11-Peptacosyl octanoate	1.57 (0.43)	64.78 (40.59)	32.32 (3.16)	1351.37 (320.48)	32.50	34	127, 145, 350/1	493
61	11-Tetracosyl decanoate			0.54 (0.11)	22.7 (9.21)	33.20	34	155, 173, 336/7	507
62	11-+13-+15-Methyltritriacontane	0.54 (0.26)	21.27 (13.22)			33.21	34	168/9, 196/7, 224/5 252/3, 280/1, 308/9	477
63	11,15-Dimethyltritriacontane	8.78 (2.50)	360.6 (225.93)	2.94 (1.73)	120.9 (84.78)	33.40	35	168/9, 238/9, 280/1, 350/1	491
64	Trimethyltritriacontane	0.59 (0.32)	21.53 (12.95)			33.60	36		505
65a	11-Pentacosyl decanoate	0.82 (0.4)	39.23 (38.86)	5.25 (0.78)	219.05 (54.41)	33.93	37	155, 173, 350	521
65b	Dimethyltritriacontane			1.25 (0.85)	52.36 (41.06)	34.05	35		491
66	Dimethyltetratriacontane	0.68 (0.29)	28.51 (18.82)			34.30	36		505
67	13,17-Dimethylpentatriacontane	1.14 (0.49)	47.83 (34.29)			35.31	37	196/7, 252/3, 266/7 350/1	519
68	Dimethylpentatricontane	3.13 (0.93)	128.21 (76.76)			35.42	37		519
69	Dimethylheptatriacontane	2.71 (1.87)	113.02 (86.72)			37.33	39		547
70	Dimethyloctatriacontane	4.20 (4.09)	185.79 (179.56)			39.16	40		561

"Compounds expressed as percentages and quantities in nanograms per milligram of insect weight, with standard deviation (SD) in parenthesis.

of insect weight), and branched alkanes with one or more methyl groups. Male *Diglyphus* contain 19% monomethylalkanes (0.8 μg/mg of insect weight), 46% dimethylalkanes (1.9 μg/mg of insect weight), and 4% trimethylalkanes (0.2 ng/mg insect weight).

Extracts obtained from females (mean weight ≅ 0.1 mg) contained the same hydrocarbons as males but in different proportions (Table 2). Hydrocarbons accounted for only 29% of the cuticular mixture. The largest group was *n*-alkanes, which accounted for half of the hydrocarbons (16%, or 0.7 μg/mg of insect weight). In males the largest group of hydrocarbons was dimethylalkanes.

The most pronounced difference between males and females involved nonhydrocarbon compounds, which accounted for the remaining 70% of the cuticular extract in females, (2.9 μg/mg of insect weight) versus 14% in males (0.8 ng/mg of insect weight). Peak 47a, one of the three major nonhydrocarbon compounds (>5% of the total mixture) accounts for 31% (about 1.3 μg/mg of insect weight) of the female cuticular mixture, peaks 58–60 account for 32%, and peak 64 for 5%.

GC-MS analysis of the nonhydrocarbon fraction obtained from females revealed a series of bis-homologous compounds whose mass spectral data did not fit with the classic fragmentation scheme for *n*-esters of Attygalle et al. (1987). The EI spectrum of the first major compound (peak 47a) showed the highest visible fragment at *m/z* 322 ($C_{23}H_{46}$ upon high resolution mass spectrometry) and a particularly intense signal at *m/z* 127 ($C_8H_{15}O$). While the alkene fragment was regarded as a result of a MacLafferty rearrangement, the oxygen-containing fragment together with a doublet of low intensity at *m/z* 144/145 pointed to octanoic acid as a partial structure (Tengö et al., 1985). With chemical ionization, the fragmental ion was strong, whereas the molecular ion 465, which corresponds to $(M-1)^+$, was weak. These findings are consistent with an ester with 31 carbon atoms: 23 at the alcohol side and 8 at the acid side. Comparison of the spectra of the natural compounds and synthetic compounds with a straight-chain primary alcohol showed no concurrence (the molecular ion was present and very clear in EI). According to an earlier work (Tengö et al. 1991), the target compounds did not represent classic wax-type esters. As a result, we identified esters of octanoic and decanoic acids, with secondary alcohols that have a pronounced acyl/protonated-acid signal, especially when the alkoxy moiety is close to the middle of the chain (Blomquist et al., 1972; Tengö et al., 1985), with a chain length from 21 to 25 carbons atoms. Nine esters were identified in females as compared to only six (with two in trace amounts) in males. The three absent esters in males were the two with 24 carbon atoms (peaks 52c and 61), and peak 52a (a docosyl decanoate) (see Table 2).

Reduction of the crude nonhydrocarbon fraction with lithium alumina hydride yielded 1-octanol and 1-decanol (as determined by their spectra using

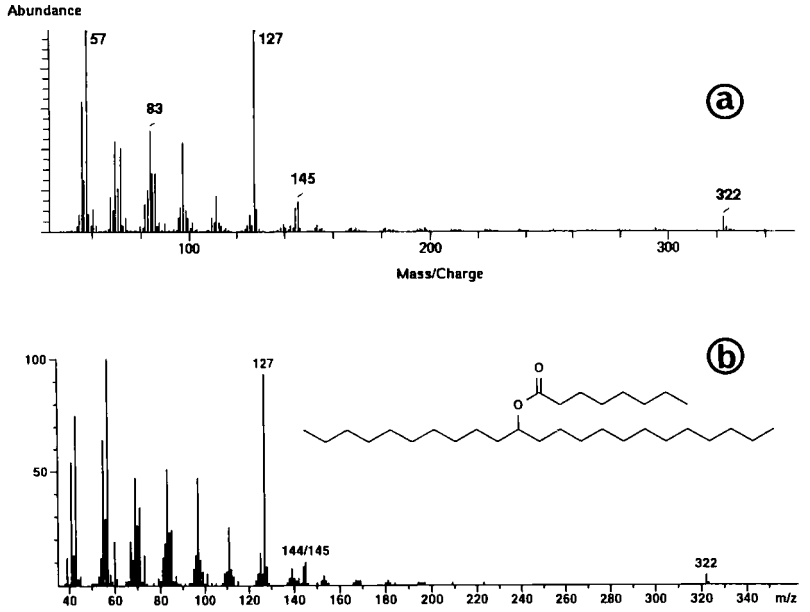

FIG. 3. Mass spectrum of the natural 11-tricosyl octanoate (a) and its synthetic compound (b).

authentic reference samples) and 11-tricosanol along with small amounts of 11-heneicosanol and 11-pentacosanol. 11-Docosanol and 11-tetracosanol were not observed, probably because these products accounted for less than 1% of the mixture. A synthetic sample of 11-tricosyl octanoate produced the same mass spectrum as the natural product, coeluted upon gas chromatography (Figure 3), and yielded the same key fragments as those reported by Blomquist et al. (1972) and Warthen and Uebel (1980). The same was true for the other 11-alkanol esters. Attempts to determine the absolute configurations of the esters failed, due to the great similarity of the alkyl chains side of the functional group. Further ethological tests with pure synthetic enantiomers are needed to establish the absolute configuration of the natural pheromones.

DISCUSSION

This study demonstrates that in *Diglyphus isaea* recognition of a female by a male occurs at close range, i.e., within a few millimeters. Recognition appears to be based mainly on chemical signals, and the role of visual signals was negligible in our experimental conditions. The fact that the male no longer

detects a female washed with hexane proves that the signal triggering male courtship behavior is on the female cuticle. This is further confirmed by the fact that the compounds involved can be extracted and transferred to a lure. Based on observations of male behavior, we speculate that contact pheromones are involved.

Our results in *Diglyphus isaea* indicate that the principal compounds inducing male courtship behavior are contained in the nonhydrocarbon fraction of the female cuticular extract. With regard to nonhydrocarbon compounds, the cuticular lipid composition is more varied quantitatively and qualitatively in females than in males. These substances appear on images upon emergence and do not change during adulthood. In a previous study (Finidori-Logli, 1994), we did not observe these oxygenated products in *Diglyphus* nymphs. This finding contrasts with results in *Melanoplus differentialis*, in which ester levels are high in last-instar nymphs but low in adult females (Warthen and Uebel. 1980). Lockey (1985) noted the presence of oxygenated compounds in 19 species of different genera. In this regard, it has been observed that the quantity of oxygenated compounds in the cuticle varies according to developmental stage in some species (Danehower and Bordner, 1984; Backer et al., 1979).

Since the method used to collect the insects did not rule out contact between the two sexes before collection, the possibility that the nonhydrocarbon products observed on the cuticle of males were acquired by contact with females cannot be ruled out. The chemical dimorphism that we observed between male and female, due essentially to long-chain esters, has been observed previously by other groups (Warthen and Uebel, 1980; Warthen et al., 1981). The latter authors were the first to suggest that sex recognition was based on differences in the cuticular mixture of long-chain esters and hydrocarbons.

In insects the cuticle is coated by lipids, the largest group being hydrocarbons (Lockey, 1988, de Renobales et al., 1991). In addition to the well-known protective role of lipids especially against desiccation (Hadley, 1981), there is now growing evidence that they also provide chemical behavioral signals (Howard, 1993). A few studies are available concerning cuticular hydrocarbons of parasitic Hymenoptera (Takashashi et al., 1990; Howard, 1992; Howard and Liang, 1993; Bagnères et al., 1996). In *Diglyphus isaea*, the hydrocarbon composition of the cuticle is sex specific. The male cuticle is extremely rich in branched alkanes, while the female cuticle is composed almost exclusively of *n*-alkanes and 3-methylalkanes.

Contact sex pheromones whose biosynthetic pathways are now better understood (Schal et al., 1994), especially regarding those of cuticular origin, seem to be under hormonal control (Wicker and Jallon, 1995a,b). In *Drosophila*, a portion of antennal lobe and mushroom bodies are involved in olfactory processing and may function in the recognition of contact sex pheromone and in control of sex-specific behavior (Ferveur et al., 1995).

The quantity of oxygenated derivatives of hydrocarbons on the cuticle is low (Lockey, 1988). The most common nonhydrocarbon products are primary fatty acids, alcohols, esters of these acids, ketones, and aldehydes. On the insect cuticle, secondary alcohols, esters of secondary alcohols, hydroxylated acids, diols, steroids, and terpenes are less frequent, but long-chain wax-type esters are not so uncommon (Blomquist and Jackson, 1979; Jackson, 1981; Buckner, 1993). Blomquist and Jackson (1973) found that in *Melanophus sanguinipes* *n*-alkanes were hydroxylated to secondary alcohols, and their esterification took place on the surface layer. Esters are typical constituents of exocrine secretions of many Hymenoptera, especially bees, and are frequently found on the surface layer (Francke, 1986; Francke et al., 1984). Long-chain esters (e.g., *n*-octadecyl hexadecenoate) have been described in mixtures with hydrocarbons in the Dufour gland of parasitic bees (Sick et al., 1994). This is the first report of secondary wax esters with 11-alcohols and short-chain acids. To the best of our knowledge, these alkyl-alkanoate compounds have only been described in species of *Melanoplus* (Buckner, 1993) and, unlike *Diglyphus*, were made up of acids with longer chains (between 10 and 20 carbons).

Our experience also indicates that tactile feedback plays a role early in courtship. While the chemical composition of the cuticle allows the male to recognize the female, it is by movements, especially of the antenna, that he is able to determine if the female is receptive. This mode of female recognition, obtaining consent after contact, has also been described in braconids (Matthews, 1974), and in particular in *Ascogaster reticulatus* (Kainoh, 1986), but never in Eulophidae.

While our findings help to explain how sex partners recognize each other at close range, the question of long-range recognition remains open. Several factors suggest that long-range recognition is necessary. Males emerge before the females and usually do not stay on the leaves from which they emerge. Females begin searching for a host within the first hour after emergence. Leaving long-distance recognition to chance would not only be a waste of time but also jeopardize procreation. Thus, there must be factors that promote encounters and ensure reproduction in parasitoids.

One possibility is that encounters result from intense searching at the side of the prey, i.e., at the plant area. We have not studied this hypothesis, but a very interesting finding reported from in-cage studies (Finidori-Logli, 1994) suggests a possible answer. Finidori-Logli et al. (1996) reported that a male that demonstrates no interest in an isolated female at long range was attracted to the same female when she was placed on a bean plant leaf. This attraction was even stronger if the leaf had been mined. This suggests that the male uses odoriferous signals from the plant to locate the area where he has the best chance of finding a female. Thus, encounters between the sexes in *Diglyphus isaea* could depend on a close-range signal involving cuticular contact pheromones,

as demonstrated in the present study, and on a long-range perception of volatile compounds from the host plant.

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HYDROCARBONS OF *Nasutitermes acajutlae* AND COMPARISON OF METHODOLOGIES FOR SAMPLING CUTICULAR HYDROCARBONS OF CARIBBEAN TERMITES FOR TAXONOMIC AND ECOLOGICAL STUDIES

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Abstract—Using data from the arboreal nesting *Nasutitermes acajutlae* (Holmgren), we propose standard collection and extraction methodology for characterization of cuticular hydrocarbons of termites under field conditions in the tropics. Specifically, we evaluated: (1) the effect of the duration and the number of extractions; (2) the effect of drying termites before extraction; (3) the effect of sample size; (4) the effect of solvents (ethanol versus hexane) on cuticular hydrocarbon profiles. Olefins comprise ca. 70% of the cuticular hydrocarbons of *N. acajutlae*. Hydrocarbons consist of two distinct groups: early-eluting components, primarily *n*-alkanes and methyl-branched alkanes, and late-eluting compounds, which consist almost exclusively of unsaturated components with one to six double bonds. Soldiers have more early-eluting compounds than workers or alates. Nests from the same island had qualitatively similar, but quantitatively dissimilar hydrocarbon mixtures. Brief extractions of 300 live workers in 10 ml of hexane for only 20 sec produced a hydrocarbon mixture equivalent to a 10-min extraction. Long-term extraction of 300 workers in hexane for two years resulted in different mixtures of hydrocarbons. Drying workers tended to enhance extraction of the less abundant unsaturated compounds such as $C_{41:4}$ and $C_{41:5}$. A single extraction of a

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minimum of 100 workers (live or dried), with hexane for 20 sec to 10 min is best; these extraction regimes resulted in mixtures of hydrocarbons that are quantitatively very similar. For quantitative comparisons, extracts from dried samples should not be compared to those from live samples. Storage in ethanol caused numerous unidentified, nonhydrocarbon compounds to be extracted either from the cuticle or from internal tissues.

Key Words—*Nasutitermes acajutlae*, chemotaxonomy, Isoptera, Termitidae, tropical termites, gas chromatography, cuticular hydrocarbons, olefins, mass spectrometry.

INTRODUCTION

Termites play an essential role in the ecological dynamics of many tropical ecosystems, recycling nutrients and aerating soils in forests, mangrove swamps, and grasslands. Some species of termites are also economically important as structural pests in urban, as well as rural, areas of the tropics. The termite fauna of tropical regions is known to be diverse, but species diagnosis remains equivocal in many groups. Cuticular hydrocarbons are useful for discriminating termite species in tropical (Haverty et al., 1990b, 1991a, 1992, 1997; Howard et al., 1988) and temperate (Haverty et al., 1988, 1991a; Howard et al., 1978, 1982a,b; Thorne and Haverty 1989; Thorne et al., 1993) regions. Species sorting and diagnosis based on such chemical separation may then be used to facilitate discovery of morphological criteria for discriminating species (Haverty et al., 1988; Thorne and Haverty, 1989) and delimiting geographic distributions (Thorne et al., 1993).

Comparative research for characterizing cuticular hydrocarbons for taxonomic or ecological studies will necessitate uniform protocols among investigators. Field circumstances must also be considered. In the tropics, termites are usually collected far from laboratory facilities. Hydrocarbon extractions must therefore be delayed until well after the insects have been collected. Standard preservation techniques, such as freezing, are usually impractical. Keeping subcolonies, groups, or samples of a specific size alive and healthy for more than a few hours is very difficult. Hot sun and predacious ants usually render field samples unusable, and live cultures are prone to problems with humidity, food stress, and pathogenic fungi. Collection of specimens directly into 70% or 85% ethanol may partially extract hydrocarbons or include some debris and potential contaminants. Long-term retention of specimens in ethanol or in a solvent such as hexane for extended periods, although potentially quite convenient, may also extract internal hydrocarbons and confound the characterization of cuticular hydrocarbons.

We have collected termites from the islands in the Caribbean and have also received specimens collected by colleagues, for characterization of cuticular

hydrocarbons. The method of collection has varied among these efforts. To interpret these data we must know if different collection/extraction regimes are equivalent or comparable. We also want to recommend the most appropriate technique for a given taxon to collaborating scientists.

In the earliest studies of termites that introduced the concept of species specificity of cuticular hydrocarbons, it was not specified how the insects were handled before extraction (Howard et al., 1978, 1982a). In later studies of chemical mimicry by termitophilous staphylinids, Howard et al. (1980, 1982b) separated the termitophiles from the termites and froze the beetles at −20°C before extraction. Haverty et al. (1988) froze *Zootermopsis* spp. individuals, then subsequently thawed them to room temperature before extraction. The termites were brought from the field to the laboratory alive (Haverty et al., 1988). However, after extracting a few live termites, one of us (L.J.N.) noticed that the termites convulsed and emptied their gut contents into the hexane during the process. Freezing the insects, followed by extraction of the specimens at ambient temperature, prevented this possible source of contamination.

Subsequent studies of cuticular hydrocarbons of termites have used live termites extracted in the field or in the laboratory [Howard et al., 1988 (for *Nasutitermes*); Haverty et al., 1990b (for *Nasutitermes*), 1991a, unpublished (for *Reticulitermes*)], or termites dried in the field and later extracted in the laboratory [Haverty et al., 1990a, 1996b (for *Coptotermes*), 1991a, 1992 (for *Reticulitermes* and *Coptotermes*)]. Our preliminary results comparing extraction of live versus dried *Nasutitermes acajutlae* (Holmgren) indicated that the resulting hydrocarbon mixtures were not equivalent.

Many of our colleagues find it difficult to dry termites while collecting termites in the tropics because of logistical problems. Ovens, heating lamps, and electricity are not always available. Since drying is often impractical, many researchers prefer to collect termites directly into alcohol or hexane. Collectors also do not want to divert their efforts away from maximizing the number of samples they can collect while in the field. The usual field technique is to place termites directly into 80% or 100% ethanol for later transmittal to the museum or laboratory. Detailed comparisons of cuticular hydrocarbon patterns derived from extractions of live or dried termites or termites stored for extended periods in ethanol or hexane are thus warranted and are one focus of this study.

Another factor affecting the assessment of the cuticular hydrocarbon pattern is the number of insects that are extracted or quantity of hydrocarbon extracted. In some studies a variable number of individuals (and mixture of castes) was included. Haverty et al. (1991a) used from 15 to 200 *Reticulitermes* spp. workers per sample in their preliminary study of this genus. In most of our studies an exact number of termites was extracted: individual *Zootermopsis* spp. pseudergates, nymphs, soldiers, or alates (Haverty et al., 1988); 100 *Coptotermes formosanus* Shiraki workers or soldiers (Havery et al., 1990a); 200 *C. formo-*

sanus workers or 50 soldiers (Haverty et al, 1996b); and 100 *Nasutitermes costalis* (Holmgren) or *N. ephratae* (Holmgren) large workers (Haverty et al., 1990b).

Extraction of too few individuals can result in a diluted extract and require a deviation from a standard technique. Characterization of hydrocarbons from dilute extracts would likely underestimate or not detect the less abundant compounds and overestimate the abundant compounds. We know of no published studies to determine the minimum number of individuals necessary to characterize the cuticular hydrocarbons of any termite taxon.

One of the primary reasons we began studies of extraction methodology was to remedy a problem encountered during studies of the cuticular hydrocarbons of *N. corniger* (Motchulsky), *N. ephratae*, and *N. costalis* (Haverty et al., 1990b; Howard et al., 1988). Haverty et al. (1990b) experienced difficulty obtaining a sufficiently large hydrocarbon sample by extracting 100 large workers of *N. costalis* or *N. ephratae* in the field in Trinidad. The samples taken during that study were barely sufficient to allow quantification of the hydrocarbon mixtures. Thus, we designed a first set of experiments in 1989 to better understand the difficulties experienced with the characterization of cuticular hydrocarbons from *N. costalis* and *N. ephratae*.

Given that any taxonomic or ecological study entails processing a large number of samples, it is essential to keep the method of analysis as consistent as possible. We have developed chromatographic conditions and mass spectral acquisition parameters that give optimal results for the vast majority of insect samples. We therefore focused our efforts on comparing various collection and extraction regimes using our standard analytical technique.

In the present study, we attempted to define a standard methodology for collecting and extracting termites under field conditions. Our field work was based at our research site on Guana Island, British Virgin Islands (BVI) (Thorne et al., 1994; Haverty et al., 1997). This island has a diverse termite fauna consisting of nine species in three families. The most conspicuous, and apparently abundant, species is the arboreal nesting *N. acajutlae* (Thorne et al., 1994; Scheffrahn et al., 1994). This species, as well as a closely related species, *N. nigriceps* (Holmgren), is numerically and ecologically conspicuous on many of the Caribbean islands (Thorne et al., 1994). A better understanding of the appropriate procedure(s) for sampling and extracting cuticular hydrocarbons of *N. acajutlae* is important to our understanding of the taxonomy, ecology, and biogeography of this and other *Nasutitermes* species. The methodology described here is optimal for sampling and extracting cuticular hydrocarbons for this species and should be applicable to most termites in the tropics. Very small termites, such as *Parvitermes* spp., will likely require larger samples, whereas the larger termites, such as *Neotermes* spp. and *Incisitermes* spp., will require fewer individuals.

For taxonomic studies we consider cuticular hydrocarbons useful primarily for separating species within a genus; obvious and consistent morphological characters usually suffice to separate taxa at higher levels (Haverty et al., 1991b). Hydrocarbons beneficial for taxonomic studies should be abundant, not minor components (at least 1.0%, but preferably 5.0% of the total hydrocarbon component). However, if a hydrocarbon is consistently detected, even in quantities below 1.0%, we would consider it a useful taxonomic character. Useful hydrocarbons should also be unique or present in only a few of the species, or conversely, they should be common in most of the species yet completely absent, rare, or of insignificant quantities in one or a few species. Furthermore, they should have a unique elution time so that they do not coelute with another hydrocarbon in the same species, nor should they elute at a time similar to that of a different hydrocarbon in a different species (Haverty et al., 1991b). From a set of hydrocarbons with these characteristics, dichotomous keys can be constructed. For ecological or biogeographic studies of the same species, examinations of the quantitative differences of the hydrocarbon mixture would be of paramount importance.

In this paper we report the results of studies conducted in 1989 and 1993 to compare and improve our sampling and extraction techniques for the characterization of cuticular hydrocarbons of tropical termites. In 1989 we examined influences of the number and duration of extractions. In 1993 we tried to design studies that would ultimately provide us with an optimal field method for characterizing both the composition and relative abundance of the cuticular hydrocarbons of *N. acajutlae*. We compare methodologies and suggest standard and alternative, acceptable methodologies for both chemotaxonomic and ecological studies of this termite. Specifically, we evaluate: (1) the effect of the duration and the number of extractions; (2) the effect of drying termite specimens before extraction; (3) the effect of sample size; and (4) the effect of solvents (ethanol versus hexane) on the reproducibility of cuticular hydrocarbon profiles.

METHODS AND MATERIALS

Collecting and Sorting Termites. We collected workers and soldiers of *N. acajutlae* during two separate trips (1989 and 1993) to Guana Island, BVI. During each trip different collection techniques were used. In 1989 two nests were sampled near White Sands Beach. Portions of nest material containing workers and soldiers were returned to the laboratory. Nest material was placed in pans and folded cardboard index cards were placed on top of this material. Workers and soldiers climbed onto the cards and then were tapped off into trays, where they were sorted, counted, and prepared for hydrocarbon analyses as described below.

In 1993 we selected 13 colonies from the same area, and we used a modification of a technique demonstrated to us by Dr. Jan Krecek. This is the best technique for collecting large numbers of workers and soliders with the least effect on nest structure. A tangential slice, 2–3 cm deep and 15–20 cm in diameter, was removed from the more fragile exterior portion of a nest and discarded. Over this breach we placed one or two 20-cm × 20-cm squares of moist corrugated cardboard. If we were able to cut the slice from the top of a nest, the corrugated cardboard squares were secured against the surface of the nest with a stone. If the slice was taken from the side of the nest (often we could not reach the top of a nest) the corrugated cardboard squares were secured to the nest with 7.5 cm, galvanized, finishing nails.

As soon as the slice of nest was removed and the corrugated cardboard squares put in place, soldiers swarmed out of the breach and covered the squares; workers immediately retreated into the nest. In less than 60 sec the squares were removed and a collection of nearly pure soldiers was tapped into a collection pan. To readily obtain a nearly pure sample of hundreds to thousands of soldiers, this process can be repeated several times. We then visually scanned all individuals in the collection pan and removed the few workers in the sample.

Workers were collected by leaving the moist squares of corrugated cardboard on the nest for up to 60 min. Once the alarm reaction of the soldiers began to dissipate, workers ventured to the underside of the squares and began to repair the breach. When the cardboard was gently removed, a dense sample of workers adhered to its surface. In contrast to the technique for collecting soldiers, the squares of corrugated cardboard containing the workers (and relatively few soldiers) were placed in a collection pan and returned to the field laboratory for sorting, counting, and preparation of workers for hydrocarbon analyses as described below.

Preparation of Termites for Extraction. Because soldiers squirt glue over the containers and themselves, they were difficult to count individually. For the purposes of cuticular hydrocarbon analyses, we measured ca. 5–8 ml of soldiers (about 500 individuals) in 20-ml scintillation vials (Wheaton scintillation vials, foil-lined caps) for extraction or drying. Workers were separated and individually counted into 20-ml scintillation vials for extraction or drying of the appropriate number of individuals. Alates were occasionally present; when collected, they were individually counted into vials and handled in exactly the same way as the workers.

Additional termites from each of the sampled colonies (with soldiers and alates, when available, as diagnostic castes) were placed in 80% ethanol (from Quantum Chemical Corp., 200-proof punctilious dehydrated alcohol) to serve as voucher specimens. These voucher specimens are kept at the Pacific Southwest Research Station, Albany, California, and the Department of Entomology, University of Maryland, College Park, Maryland.

Standard Extraction Process. Cuticular lipids were extracted by immersing termites in *n*-hexane (EM Science OmniSolv, suitable for HPLC, GC, and residue analysis). Our usual procedure has been a 10-min extraction of 100 termites in 10 ml of *n*-hexane. In this study, we used this procedure as the standard to evaluate the various extraction regimes described in the following sections. The lipid extracts resulting from each of the various methods were pipetted through 4 cm of activated BioSil-A (silica gel, BioRad Laboratories, 100–200 mesh) in Pasteur pipet minicolumns (5 mm ID) in order to isolate the hydrocarbon components. The resulting hydrocarbon extracts were evaporated to dryness under nitrogen and redissolved in 60 μl of *n*-hexane for gas chromatography–mass spectrometry (GC-MS) analyses.

Duration and Number of Extractions. In 1989 we evaluated the following extraction regimes using *N. acajutlae* from Guana Island, BVI, to determine whether we could improve upon extracting 100 live workers in 10 ml of *n*-hexane for 10 minutes:

A. Extract 300 workers in 10 ml *n*-hexane for 10 min (standard technique with an increased sample size).

B. Extract 300 workers three times in 10 ml of *n*-hexane for 10 min, keeping each extract separate to determine if the standard technique left significant quantities of hydrocarbon on the sample.

C. Extract 300 workers in 10 ml of *n*-hexane for 20 sec followed by a 10-min extraction in 10 ml of *n*-hexane, keeping each extract separate to determine whether a brief extraction would produce a mixture of hydrocarbons comparable to the standard, A, extraction.

D. Extract 300 workers in 10 ml of *n*-hexane for 20 sec followed by extracting for 24 hr in 10 ml *n*-hexane, keeping each extract separate (same as extraction B with an extended second extraction) to determine whether an extended extraction would remove additional hydrocarbons from the cuticle or from other tissues.

E. Extract 300 workers in 10 ml of *n*-hexane for 24 hr to allow for a less stringent extraction schedule.

F. Long-term extraction of 300 workers in 10 ml of *n*-hexane for two years to allow field collection with subsequent laboratory storage for an extended period.

Effect of Drying. In 1993 six samples of 100 *N. acajutlae* workers from each of 13 nests were allocated to evaluate the effect of drying termites prior to extraction. The cuticular hydrocarbon profiles of the dried termites were compared to those generated from an extraction of live termites. Live termites were extracted at the Guana Island, BVI, field laboratory by placing each of three samples from each colony directly into separate 20-ml scintillation vials and extracting these samples in 10 ml of *n*-hexane for 10 min (standard procedure A). The hexane from each of 39 vials was then decanted into separate 20-ml

scintillation vials and subsequently returned to our laboratory in California for purification and analysis of cuticular hydrocarbons.

The other three samples of *N. acajutlae* workers from each of 13 colonies were dried by placing 20-ml scintillation vials, each containing 100 individuals, in a wire box over a single 75-W, reflecting, incandescent light. The amount of time required to completely dry termites varied slightly as a function of the position of the vials over the bulb. The position of the vials over the bulb was changed in an attempt to make the drying time similar for each sample. Once termites were completely dried, we attempted to keep them dry by tightly sealing the vials. The vials were returned to our laboratory in California for extraction, purification, and analysis of cuticular hydrocarbons. Until they were extracted, dried samples were kept in a freezer at −20°C in California to prevent possible fungal growth.

Effects of Sample Size. In 1993, samples of 25, 50, 100, or 200 *N. acajutlae* workers from five different colonies were extracted either live or dried as described above. Each combination (sample size × live versus dried × colony) was replicated three times.

Effects of Solvent. In 1993, for each of five colonies, three replicates of 200 workers were placed in 20 ml of 100% ethanol and left in the solvent for 60 days. The ethanol extract was decanted and evaporated to dryness under nitrogen. The extract was then redissolved in 10 ml of hexane and processed by the standard procedure. After the ethanol was drained from the termites, the insects were dried and extracted with hexane following the standard procedure.

Characterization of Cuticular Hydrocarbons. Each sample was analyzed by GC-MS in order that the presence of all compounds could be verified by mass spectral data. These data allowed us to obtain information about coeluting compounds in particular. GC-MS analyses were performed on a Hewlett-Packard (HP) 5890 gas chromatograph equipped with a HP 5970B Mass Selective Detector interfaced with HP Chemstation software. The GC-MS was equipped with an HP-1, fused silica capillary column (25 m × 0.2 mm ID) and operated in split mode (with a split ratio of 8:1). A 3-μl aliquot was injected into the GC-MS. Each mixture was analyzed using a temperature program from 200°C to 320°C at 3°C/min with a final hold of 16 min. Electron impact (EI) mass spectra were obtained at 70 eV. *n*-Alkanes were identified by their mass spectra. Mass spectra of methylalkanes were interpreted as described by Blomquist et al. (1987) to identify methyl branch locations. Alkenes were identified by their retention times relative to *n*-alkanes and/or mass spectra; the latter only for olefins with 35 or more carbons. A typical alkene mass spectrum shows a molecular ion and a series of fragments at 14-mass unit intervals (e.g., 69, 83, 97), similar to those displayed by *n*-alkanes, less 2 mass units. Interpretation of the mass spectra of dienes and polyunsaturated hydrocarbons was extrapolated from this pattern, i.e., for each double bond, the molecular ion is decreased by 2 mass units.

In the text and tables, we use shorthand nomenclature to identify individual hydrocarbons or mixtures of hydrocarbons. This shorthand uses a descriptor for the location of methyl groups (X-Me), the total number of carbons (subscript XX) in the hydrocarbon component excluding the methyl branch(es), and the number of double bonds following a colon (subscript Y). Thus, pentacosane becomes *n*-C_{25}; 11-methylpentacosane becomes 11-MeC_{25}; and pentacosene becomes $C_{25:1}$. Hydrocarbons are presented in the tables in the order of elution on our GC-MS system.

Integration of the total ion chromatogram was performed by the data analysis software (HP59974J Rev. 3.1.2) in the HP Chemstation. GC-MS peak areas were converted to percentages of the total hydrocarbon fraction. These percentages for each hydrocarbon peak were the response variables used to make statistical comparisons among preparation and extraction techniques.

Statistical Analyses. The effect of drying termites before extraction was assessed by a series of *t* tests of the differences between the mean percentage of a given hydrocarbon (three samples from each colony were averaged, which resulted in 13 replicates, one for each colony) from the two conditions (Steel and Torrie, 1960). The significance of the calculated *t* value was tested at $\alpha = 0.00227$ [0.05/22, the number of consistently abundant (0.3% or greater of the total) hydrocarbon peaks for workers]. Our null hypothesis was that the 10-min extraction of 100 dried termites was not significantly different from the standard 10-min extraction of 100 live termites.

The effect of group size was tested with an analysis of variance for each hydrocarbon (Steel and Torrie, 1960). Each treatment combination (sample size × colony) was replicated three times. The four sample sizes were compared separately for termites extracted live or dried. Our null hypothesis was that all sample sizes provide extracts with the same percentages of each hydrocarbon. The significance of the *F* statistic was also tested with $\alpha = 0.00227$. Significant differences among mean percentages for each hydrocarbon were determined by Tukey's honestly significant difference (HSD) procedure. We were looking for the smallest sample size that results in a hydrocarbon profile equivalent in percentage of hydrocarbons to that of the next greatest sample size. A sample size was considered inadequate if it yielded a hydrocarbon mixture that had significantly different percentages from that of the next larger sample size.

RESULTS AND DISCUSSION

Cuticular Hydrocarbons of N. acajutlae

We identified 33 hydrocarbons from workers, 45 from soldiers, and 43 from alates of *N. acajutlae*; a total of 60 different hydrocarbons or isomeric mixtures in this species (Table 1; Figure 1). The hydrocarbons found in all three

TABLE 1. RELATIVE ABUNDANCE (MEAN PERCENT AND STANDARD DEVIATION) OF CUTICULAR HYDROCARBONS OF WORKERS, SOLDIERS, AND ALATES OF *Nasutitermes acajutlae* (HOLMGREN) FROM GUANA ISLAND, BVI[a]

	Workers		Soldiers		Alates	
Hydrocarbon[b]	Mean	SD	Mean	SD	Mean	SD
$C_{23:1}$	0.32	0.32	0.41	0.17	0.00	0.00
C_{23}	0.97	0.33	4.04	0.91	0.08	0.04
11-; 9-MeC_{23}[c]	0.00	0.00	0.51	0.15	0.00	0.00
3-MeC_{23} + $C_{24:1}$[d]	0.00	0.00	0.18	0.15	0.00	0.00
C_{24}	0.22	0.22	0.66	0.19	0.00	0.00
11-MeC_{24}	0.00	0.00	0.37	0.32	0.00	0.00
$C_{25:1}$	0.68	0.27	2.96	0.69	0.00	0.00
C_{25}	3.05	1.14	9.16	1.28	0.55	0.14
13-; 11-MeC_{25}[c]	0.16	0.17	1.98	0.43	0.12	0.03
5-MeC_{25}	0.00	0.00	0.00	0.00	0.07	0.01
2-MeC_{25}	0.00	0.00	0.00	0.00	0.20	0.03
3-MeC_{25} + $C_{26:1}$[d]	0.00	0.00	0.57	0.13	0.00	0.00
5,17-DimeC_{25}	0.00	0.00	0.00	0.00	0.08	0.01
C_{26}	0.26	0.27	0.59	0.16	0.14	0.02
13-; 12-; 11-MeC_{26}[c]	0.00	0.00	0.42	0.35	0.00	0.00
2-MeC_{26}	0.00	0.00	0.00	0.00	1.42	0.16
$C_{27:1}$	0.86	0.42	3.88	0.63	0.00	0.00
C_{27}	1.90	1.60	3.67	0.92	1.44	0.28
13-; 11-MeC_{27}[c]	0.07	0.11	1.14	0.21	0.00	0.00
5-MeC_{27}	0.00	0.00	0.00	0.00	0.08	0.04
2-MeC_{27}	0.15	0.13	0.14	0.09	1.67	0.29
3-MeC_{27}	0.00	0.00	0.00	0.00	0.72	0.13
C_{28}	0.15	0.19	0.13	0.12	0.30	0.08
2-MeC_{28} + $C_{29:1}$[d]	0.00	0.00	0.19	0.10	0.53	0.11
$C_{29:1}$[e]						
C_{29}	0.73	0.56	0.56	0.25	1.08	0.25
5-MeC_{29}	0.00	0.00	0.00	0.00	0.07	0.05
2-MeC_{29}	0.00	0.00	0.00	0.00	0.03	0.03
3-MeC_{29}	0.00	0.00	0.00	0.00	0.04	0.05
C_{30}	0.00	0.00	0.00	0.00	0.05	0.04
C_{31}	0.06	0.15	0.02	0.04	0.58	0.08
C_{33}	0.00	0.00	0.01	0.03	0.12	0.03
$C_{35:1}$	0.00	0.00	0.00	0.00	0.11	0.06
13-MeC_{35}	0.00	0.00	0.00	0.00	0.12	0.06
$C_{37:1}$	0.40	0.17	0.65	0.17	0.40	0.14
$C_{38:1}$	0.53	0.20	0.58	0.13	0.47	0.07
$C_{39:5}$[f]	0.36	0.33	0.28	0.25		
$C_{39:4}$[f]	2.00	0.73	1.33	0.61	3.89	0.78
$C_{39:2}$	0.43	0.48	1.29	0.56	1.14	0.11
$C_{39:1}$	20.01	2.20	15.77	2.79	14.40	1.26
15-MeC_{39}	0.04	0.12	0.36	0.27	0.00	0.00

TABLE 1. Continued

Hydrocarbon[b]	Workers		Soldiers		Alates	
	Mean	SD	Mean	SD	Mean	SD
$C_{40:5}$	0.00	0.00	0.00	0.00	0.90	0.35
$C_{40:1}$	2.51	0.42	2.89	0.62	0.00	0.00
$C_{41:4} + C_{41:5}$[g]	18.77	3.81	11.01	3.92	21.72	2.41
$C_{41:2}$	1.21	0.63	1.50	0.49	3.31	0.20
$C_{41:1}$	24.43	2.74	17.42	3.52	20.24	1.18
15-MeC_{41}	0.13	0.23	0.46	0.24	0.76	0.15
$C_{42:1}$	1.60	0.65	1.99	1.03	0.00	0.00
$C_{43:6} + C_{43:5}$[h]	4.35	1.37	2.41	1.09	6.24	1.11
$C_{43:2}$	0.83	0.52	1.14	0.43	2.63	0.27
$C_{43:1}$	10.40	1.61	7.66	1.48	10.28	1.09
15-MeC_{43}	0.00	0.00	0.00	0.00	0.43	0.11
$C_{45:5}$[e]						
$C_{45:2}$	0.00	0.00	0.00	0.00	0.32	0.11
$C_{45:1}$	2.41	0.82	1.69	0.64	3.26	0.87

[a] Three subsamples of 100 workers from each of 13 colonies. Four subsamples of ca. 4 ml of soldiers from each of 13 colonies. Two subsamples of 25–31 alates from four colonies.

[b] This shorthand (X-MeC_{XX} and $C_{XX:Y}$) uses descriptors for the location of the methyl group (X-Me), the total number of carbons (XX), excluding the methyl branch(es), and the number of double bonds (Y). Locations of double bonds are undetermined.

[c] An isomeric mixture. These monomethylalkanes coelute.

[d] This monomethylalkane and the olefin coelute.

[e] These hydrocarbons were not detected in samples from Guana Island, but were found in samples from Tortola, BVI. $C_{29:1}$ was found in trace amounts in soldiers only; $C_{45:5}$ was found in all three castes, but in amounts averaging $< 1.0\%$.

[f] These two isomers occasionally did not resolve in alates. Therefore, the areas for the two isomers were summed for alates only.

[g] These two isomers occasionally did not resolve. Therefore, the areas for the two isomers were summed.

[h] $C_{43:6}$ was identified only in alates. There were two isomers of $C_{45:1}$ that occasionally did not resolve. Therefore, the areas for the two isomers were summed.

castes segregate into two distinct groups. The early-eluting components are primarily *n*-alkanes, methyl-branched alkanes, and a few normal alkenes. The late-eluting compounds consist almost exclusively of unsaturated hydrocarbons with chain lengths of 37–45 carbons and one to six double bonds and a few monomethyl alkanes in trace amounts (especially in alates). Soldiers have considerably greater proportions of the early-eluting compounds (23–29 carbons) than do workers or alates (Table 1; Figure 1). Whereas workers and alates have at least 88% of the cuticular hydrocarbons with 31 or more carbons, soldiers have only about 69% of these late-eluting compounds. The predominant class

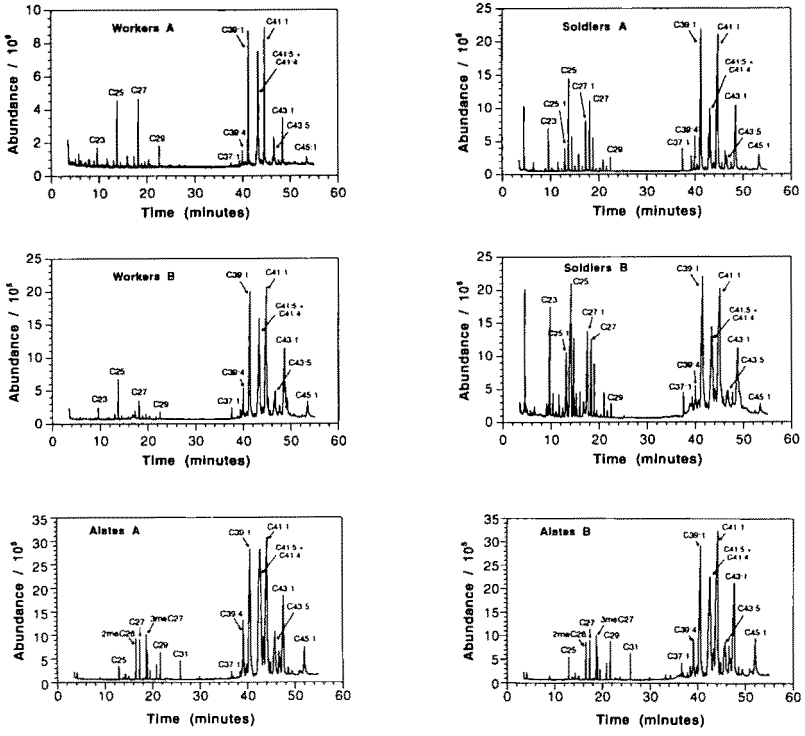

FIG. 1. Total ion chromatograms of the cuticular hydrocarbons of workers, soldiers, and alates of *Nasutitermes acajutlae* (Holmgren) from two colonies (A and B) from Guana Island, BVI. The cuticular hydrocarbon extracts were derived from dried samples of 100 workers, ca. 8 ml of soldiers, and 25 alates collected in October 1993.

of hydrocarbons in all three castes is the olefin fraction (Table 1). No dimethyl- or trimethylakanes were found in workers or soldiers.

n-Alkanes. Normal alkanes ranged from *n*-C_{23} to *n*-C_{33}; *n*-C_{25} and *n*-C_{27} were usually the most abundant. The *n*-alkanes were least abundant in alates, moderately abundant in workers, and most abundant in soldiers (Tables 1; Figure 1).

Internally Branched Monomethylalkanes. This class of hydrocarbon was nearly always encountered, in trivial amounts in workers and in significant amounts in soldiers (5.2% of the total hydrocarbon), and accounted for only 1.4% of the total hydrocarbon in alates. For soldiers most of this class of hydrocarbon eluted early, while those found in alates were in the late-eluting fraction (Table 1).

Terminally Branched Monomethylalkanes. The 2- and 3-methylalkanes occurred in trivial amounts in workers and soldiers. Alates usually had a significant component of 2-methylalkanes. These hydrocarbons were always found in the early-eluting constituents (Table 1; Figure 1).

Dimethyl- and Trimethylalkanes. Only one dimethylalkane was identified: 5,17-dimeC_{25}. It was found only in trivial amounts in alates (Table 1).

Olefins. The unsaturated component is the paramount class of hydrocarbons in the cuticular lipids of *N. acajutlae*. Olefins comprise over 90% of the total hydrocarbons in workers and alates. Because soldiers contain a larger proportion of early-eluting *n*-alkanes, the olefin component amounts to an average of 75.6% of the total hydrocarbons in this caste. For all castes, $C_{39:1}$, $C_{41:5}$, $C_{41:4}$, $C_{41:1}$, and $C_{43:1}$ combined account for >70% of the total olefin fraction (Table 1).

We are confident in our identification of the late-eluting monounsaturated alkenes. The spectra of $C_{39:1}$ and $C_{41:1}$ display the parent ion (546 and 574, respectively) as well as the characteristic fragmentation pattern with the predominant peaks of fragments (83 and 97) being 2 mass units less than would be expected from *n*-alkanes and exceeding fragment 67 in abundance (Figure 2). The polyunsaturated components, for example, $C_{41:4}$ and $C_{41:5}$, are identified by the presence of parent ions (568 and 566, respectively) which are 8 and 10 mass units less than the normal alkane with the same number of carbons (Figure 3).

Polyunsaturated alkenes have not been commonly reported from the cuticular hydrocarbons of termites. However, Moore (1969) was the first to describe the cuticular lipids from a termite, *Nasutitermes exitiosis* (Hill), from Australia. He found a complex mixture of unsaturated components with the predominant component identified as a quadrupally unsaturated, unbranched hydrocarbon, $C_{39:4}$. Similarly, we have identified high-molecular-weight dienes, trienes, and tetraenes from the cuticular lipids of *Cryptotermes brevis* (Walker), *Procryptotermes corniceps* (Snyder), *Incisitermes* spp., *Parvitermes wolcotti* (Snyder), and *N. acajutlae*, as well as other species of *Nasutitermes* from the Caribbean Basin (Haverty et al., 1992, 1997).

Effect of Duration and Number of Extractions

Early in our research on the chemotaxonomy of termites we extracted live or recently frozen individuals. The standard 10-min extraction of 300 live workers of *N. acajutlae* (Figure 4A) allowed us to resolve and characterize most of the components identified for this species (Tables 1 and 2). Additional components were identified by drying termites first (see Table 1 and *Effects of Drying* below). We also discovered that different nests of this species from the same island produced qualitatively similar (not identical), but quantitatively dissimilar, hydrocarbon mixtures (Figure 1; and unpublished observations). Further-

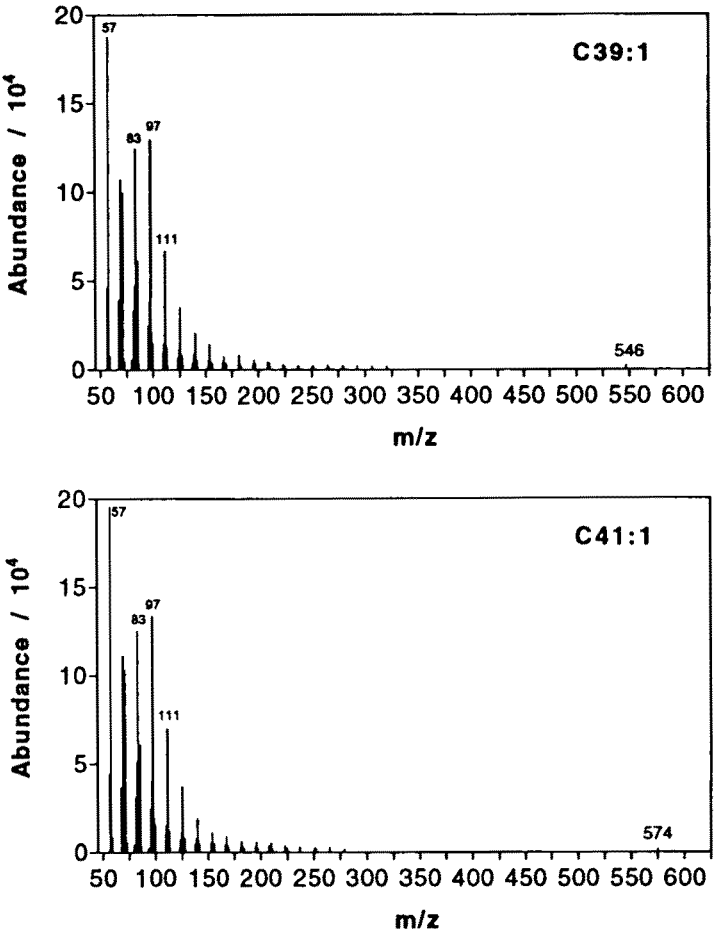

Fig. 2. Mass spectra of nonatriacontene ($C_{39:1}$) and hentetracontene ($C_{41:1}$) from cuticular hydrocarbons from workers of *Nasutitermes acajutlae* from Guana Island, BVI.

more, these colony-specific hydrocarbon analyses are repeatable; when a second group of 300 live workers was extracted using the standard procedure, the hydrocarbon mixtures from each colony were qualitatively identical and quantitatively quite similar (Figure 4A and B1; Table 2). A second 10-min extraction of the same 300 workers resulted in a hydrocarbon mixture that was quite similar to the mixture from the first extraction (Figure 4B2), although the ion abundances of the peaks were about half those in the first extract. A third, 10-min extraction resulted in an extract that did not resemble those from either of the

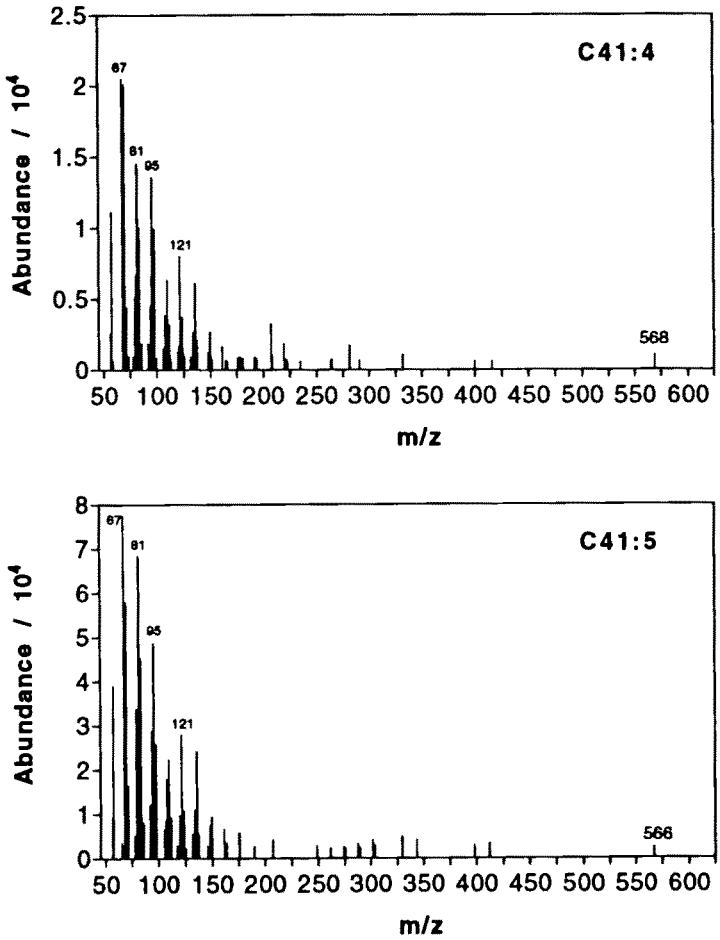

FIG. 3. Mass spectra of hentetracontatetraene ($C_{41:4}$) and hentetracontapentaene ($C_{41:5}$) from cuticular hydrocarbons from workers of *Nasutitermes acajutlae* from Guana Island, BVI.

first two extractions; only the predominant peaks (C_{25}, C_{27}, $C_{39:1}$, and $C_{41:1}$) were detected (Table 2; Figure 4B3).

An extraction of 300 live workers in 10 ml of hexane for only 20 sec appeared to be equivalent to a 10-min extraction (Table 2; Figure 4A, C1, and D1). Furthermore, the 20-sec extraction was repeatable. A subsequent 10-min extraction of the same workers produced a hydrocarbon mixture similar to that of a 20-sec extraction, although the ion abundances of the peaks were about

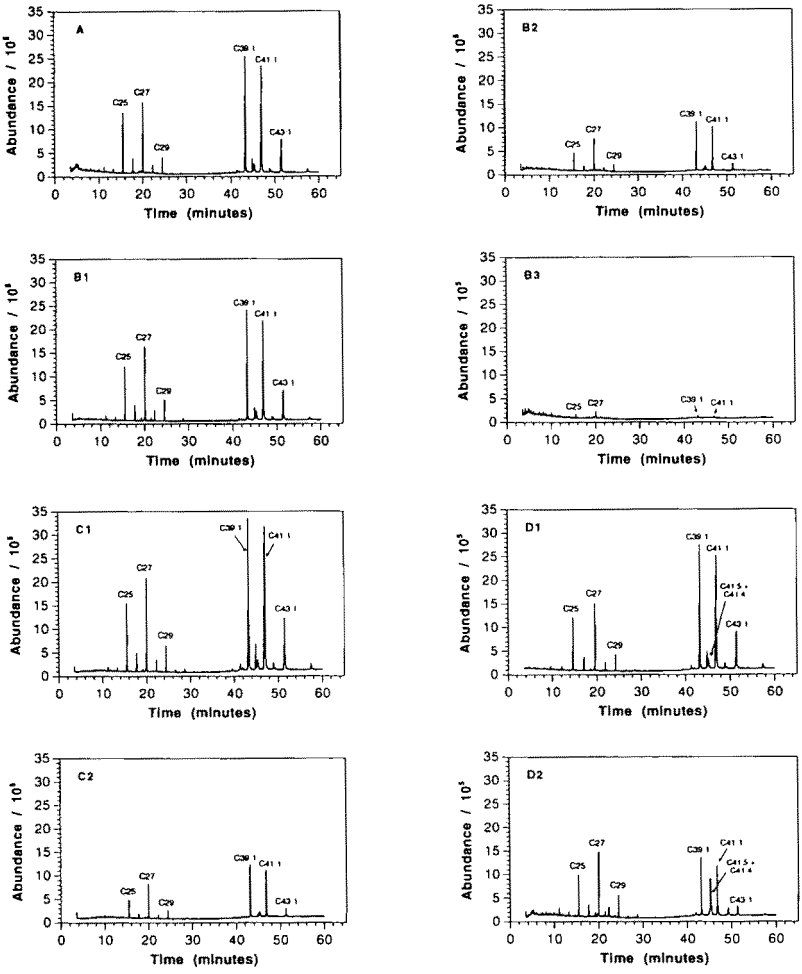

FIG. 4. Chromatograms of cuticular hydrocarbons extracted from 300 live workers from one nest of *Nasutitermes acajutlae* from Guana Island, BVI. A = extraction with 10 ml hexane for 10 min (standard); B1 = first extraction with 10 ml hexane for 10 min (equivanent to the standard); B2 = second extraction of the same termites as B1 with 10 ml hexane for 10 min; B3 = third extraction of the same termites as B1 and B2 with 10 ml hexane for 10 min; C1 = first extraction with 10 ml hexane for 20 sec; C2 = second extraction of the same termites as C1 with 10 ml hexane for 10 min; D1 = first extraction with 10 ml hexane for 20 sec (equivanent to C1); D2 = second extraction of the same termites as D1 with 10 ml hexane for 24 hr; E = extraction with 10 ml hexane for 24 hr; F = extraction with 10 ml hexane for two years. Samples A–F were each derived from independent groups of 300 workers.

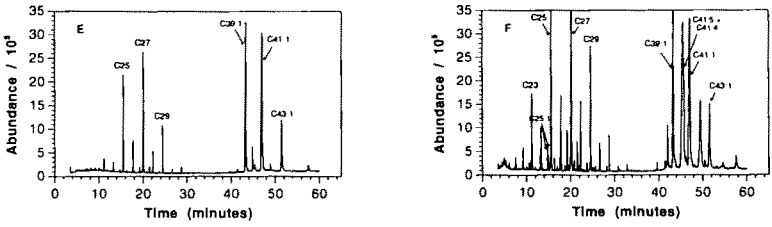

FIG. 4. Continued.

TABLE 2. RELATIVE ABUNDANCE (MEAN PERCENT FROM TWO COLONIES) OF CUTICULAR HYDROCARBONS OF WORKERS OF *Nasutitermes acajutlae* (HOLMGREN) FROM GUANA ISLAND, BVI, RESULTING FROM DIFFERENT EXTRACTION REGIMES[a]

Hydrocarbon[b]	A	B1	B2	B3	C1	C2	D1	D2	E
C_{23}	0.3	0.4	0.0	0.0	0.2	0.0	0.3	1.5	0.6
C_{24}	0.3	0.3	0.0	0.0	0.2	0.0	0.2	0.5	0.3
$C_{25\ 1}$	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C_{25}	4.7	4.7	5.3	18.4	3.5	4.9	3.9	7.5	5.6
C_{26}	0.9	1.0	0.9	0.0	0.7	0.9	0.7	1.4	1.1
2-MeC_{26} + $C_{27\ 1}$	0.4	0.3	0.0	0.0	0.1	0.0	0.2	1.2	0.5
C_{27}	5.1	6.1	7.5	20.1	4.2	7.5	4.3	9.5	6.7
C_{28}	0.4	0.6	0.8	0.0	0.4	0.8	0.4	1.3	0.7
C_{29}	1.0	1.3	1.8	0.0	0.9	1.8	0.8	3.0	1.7
$C_{37\ 1}$	0.2	0.2	0.2	0.0	0.3	0.0	0.3	0.0	0.3
$C_{38\ 1}$	0.4	0.6	0.7	0.0	0.8	0.0	0.6	0.2	0.4
$C_{39\ 4}$	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
$C_{39\ 1}$	32.0	30.0	31.7	34.3	31.3	32.6	31.4	23.0	28.4
$C_{40\ 1}$	2.7	2.9	2.4	0.0	2.9	1.6	3.0	1.8	2.5
$C_{41\ 5}$	0.7	0.7	1.6	0.0	0.7	1.2	0.7	3.9	0.7
$C_{41\ 4}$	0.9	0.9	2.4	0.0	0.9	2.3	1.5	8.0	0.7
$C_{41\ 1}$	37.1	35.4	35.9	27.2	37.7	38.2	37.2	25.9	35.3
$C_{42\ 1}$	0.9	1.1	0.4	0.0	1.2	0.0	1.2	0.0	1.0
$C_{43\ 5}$	0.0	0.1	0.0	0.0	0.0	0.0	0.4	2.0	0.0
$C_{43\ 1}$	11.2	11.2	8.0	0.0	11.8	8.3	11.3	6.3	10.9
$C_{45\ 1}$	0.6	1.5	0.4	0.0	1.6	0.0	1.7	0.1	1.4

[a] Extraction regimes A–F are outlined in the Methods and Materials and in the legend of Figure 4. Extraction procedure F was not included because of numerous unique, unknown, undetermined, or different peaks.

[b] This shorthand (X-MeC_{XX} and $C_{XX\ Y}$) uses descriptors for the location of the methyl group (X-Me), the total number of carbons (XX), excluding the methyl branch(es), and the number of double bonds (Y). Locations of double bonds are undetermined.

half those in the first extract (Table 2; Figure 4C1 and C2). Extraction of 300 workers for 24 hr after a 20-sec extraction resulted in an extract that was different from the standard extraction (10 ml for 10 min) or the 20-sec extraction. Proportional relationships changed dramatically: C_{25} and C_{27} were much more prominent as were $C_{41:4}$ and $C_{41:5}$, in extracts from the second extraction (Table 2; Figure 4A, D1, and D2).

Extraction of 300 workers for 24 hr resulted in a mixture of hydrocarbons that appeared similar to the standard 10-min extraction or to a 20-sec extraction (Table 2; Figure 4A, C1, D1, and E). Holding a sample of 300 workers in hexane for a period of two years provided a radically different mixture of hydrocarbons than the standard 10-min extraction (Figure 4F). Many hydrocarbons that we rarely see in *N. acajutlae* workers (such as C_{22}, $C_{23:1}$, $C_{24:1}$, two isomers of $C_{25:1}$, $C_{26:1}$, 11- and 13-MeC_{25}, $C_{27:2}$, 2-MeC_{26}, $C_{29:2}$, 5-MeC_{29}, $C_{31:1}$, and $C_{43:2}$) were present in quantities exceeding trace ($\bar{X} < 0.3\%$) amounts. One isomer of $C_{25:1}$ was identified in extractions of workers of *N. acajutlae* in 1993. However, the additional isomer (same mass spectrum with a different retention time) was not seen in the 1993 extractions. Furthermore, some compounds (C_{25}, C_{27}, $C_{41:5}$, and $C_{41:4}$) were present in much greater proportions. As a result of these qualitative and quantitative differences, we feel that a characteristic mixture of cuticular hydrocarbons can be obtained with extractions lasting from 20 sec to 24 hr. We do not consider the hydrocarbon mixtures from two-year extractions to be comparable to the standard 10-min extraction.

Effect of Drying

Mixtures of cuticular hydrocarbons extracted from live or dried workers were quantitatively different from one another. Of the 32 hydrocarbon peaks, the percentages of 20 of them were significantly different (Table 3). The most striking differences were exhibited in the late-eluting alkenes (Table 3; Figure 5). Drying the workers before extraction resulted in highly significant differences in the relative amounts of $C_{41:4}$ and $C_{41:5}$. Related to the apparent increased efficiency of the extraction of these compounds was the apparent decrease in the relative amounts of the most abundant compounds, $C_{39:1}$, $C_{41:1}$, and $C_{43:1}$.

In general, drying workers prior to extraction tended to enhance extraction of the less abundant unsaturated compounds. Extraction of 100 dried workers did not result in equivalent mixtures of hydrocarbons when compared to extraction of 100 live workers; these extraction regimes are not comparable for taxonomic or ecological purposes. Using either one of these extraction regimes exclusively would suffice for characterization of cuticular hydrocarbons for ecological studies, because the primary consideration would be for comparing the relative or absolute quantities of hydrocarbons. However, for taxonomic studies, the goal is to identify hydrocarbons that are consistently present even in quan-

TABLE 3. RELATIVE ABUNDANCES (MEAN PERCENT AND STANDARD DEVIATION) OF CUTICULAR HYDROCARBONS FROM SAMPLES OF 100 WORKERS OF *Nasutitermes acajutlae* (HOLMGREN) FROM GUANA ISLAND, BVI, EXTRACTED ALIVE OR AFTER DRYING

	Extracted live[b]		Extracted dry[b]		
Hydrocarbon[a]	Mean	SD	Mean	SD	t value[c]
C_{23}	0.80	0.29	0.97	0.33	−3.029
C_{24}	0.45	0.27	0.22	0.22	3.348
$C_{25:1}$	0.36	0.35	0.68	0.27	−4.445
C_{25}	2.64	0.95	3.05	1.14	−2.618
C_{26}	0.53	0.32	0.26	0.27	4.453
$C_{27:1}$	0.34	0.33	0.86	0.42	−6.610
C_{27}	1.40	0.95	1.90	1.60	−2.706
C_{28}	0.37	0.22	0.15	0.19	3.251
C_{29}	0.53	0.34	0.73	0.56	−2.438
$C_{37:1}$	0.45	0.22	0.40	0.17	1.724
$C_{38:1}$	0.63	0.25	0.53	0.20	3.537
$C_{39:4}$	0.54	0.40	2.00	0.73	−7.526
$C_{39:2}$	0.08	0.36	0.43	0.48	−3.272
$C_{39:1}$	28.12	2.49	20.01	2.20	17.314
$C_{40:1}$	2.84	0.27	2.51	0.42	3.928
$C_{41:4} + C_{41:5}$	6.23	3.61	18.77	3.81	−16.929
$C_{41:1}$	33.77	2.73	24.43	2.74	15.720
$C_{42:1}$	1.46	0.50	1.60	0.65	−1.650
$C_{43:5}$	1.01	1.13	4.35	1.37	−9.746
$C_{43:2}$	0.04	0.12	0.83	0.52	−5.876
$C_{43:1}$	13.15	1.41	10.40	1.61	11.519
$C_{45:1}$	3.44	0.66	2.41	0.82	6.233

[a] This shorthand (X-MeC_{XX} and $C_{XX:Y}$) uses descriptors for the location of the methyl group (X-Me), the total number of carbons (XX), excluding the methyl branch(es), and the number of double bonds (Y). Locations of double bonds are undetermined.

[b] Three subsamples of 100 workers from 13 colonies were either placed in a scintillation vial alive, then extracted for 10 min with 10 ml of hexane or placed in a vial, dried over an incandescent light, then extracted for 10 min with 10 ml of hexane.

[c] The critical t for $\alpha = 0.00227$ with 12 df is 3.859 for each comparison for each hydrocarbon. If $|t| > t_c$ then the difference is significant.

tities as low as 0.3% of the total hydrocarbon. Drying termites prior to the extraction greatly increases the chance of consistently detecting hydrocarbons of low abundance, especially the olefin fraction; therefore we feel dried samples should be used for taxonomy.

There is the possibility that these differences are due to the extraction of hydrocarbons from internal tissues. By definition, these hydrocarbons are not

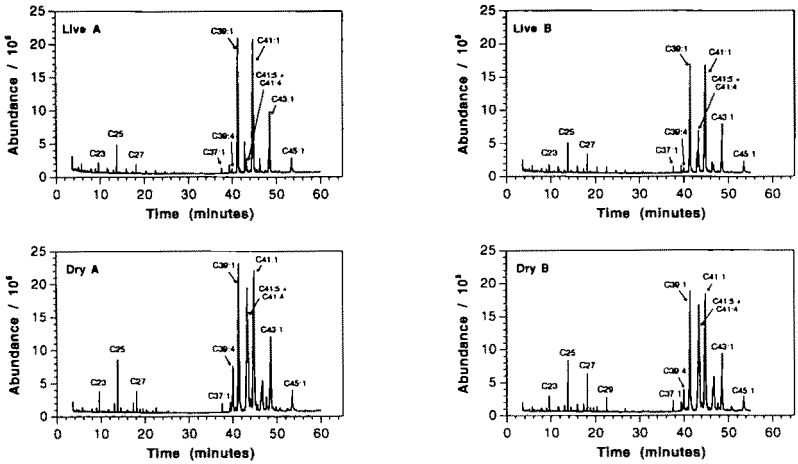

FIG. 5. Chromatograms of cuticular hydrocarbons extracted from 100 workers from two nests (A and B) of *Nasutitermes acajutlae* from Guana Island, BVI. Workers were extracted with 10 ml of hexane for 10 min either alive or after drying.

cuticular lipids, although the components may be the same (de Renobales et al., 1991). Dried termites are more fragile and often lose legs, antennae, or even heads during shipment, and also, the cuticle can become cracked. These conditions could allow the hexane to penetrate and extract lipids from the inner layers of cuticle and internal tissues. It is conceivable that some of the hydrocarbons extracted from our dried samples are not surface hydrocarbons, but those deposited on the external surface of the cuticle of the next instar (Howard et al., 1995). We found no evidence that any abundant hydrocarbons were unique to dried samples. However, the minor components $C_{41:2}$ and $C_{43:2}$ may have been unique to dried samples ($\bar{X} < 0.1\%$ in live samples; $\bar{X} > 0.8\%$ in dried samples for each component).

After examining hundreds of samples of termites extracted alive or after drying, it appears to us that the chromatograms from dried individuals are sharper and have a flatter baseline than those from live, field-extracted individuals. Three possible reasons are: (1) live termites void gut contents when placed in hexane and thus introduce contaminants, (2) live insects have a higher water content in the cuticle and less hydrocarbon is extracted because hexane is hydrophobic, or (3) hexane extracts in vials solubilize contaminants from the vial lids during transit from the field to the laboratory. The vials used were lined with foil, not Teflon. When we stored clean hexane in vials that were upside down, the resulting chromatogram had an uneven baseline similar to that resulting from extraction of live termites; however, no hydrocarbon peaks were seen. Further study of this phenomenon is warranted.

Effects of Sample Size

Statistically significant differences ($\alpha = 0.00227$) in the percentage of hydrocarbon components among sample sizes were found for 16 hydrocarbon peaks from workers extracted alive (Table 4). The most abundant components, $C_{39:1}$ and $C_{41:1}$, were not significantly different among sample sizes. Samples of 25 or 50 workers were found to be significantly different in only three cases: C_{26}, C_{28}, and C_{29}. Samples of 25 workers yielded extracts with hydrocarbon proportions significantly different from samples of 100 workers in 13 cases and from samples of 200 workers in 16 cases (Table 4). Samples of 50 workers yielded extracts with hydrocarbon proportions significantly different from sam-

TABLE 4. RELATIVE ABUNDANCE (MEAN PERCENT AND STANDARD DEVIATION) OF CUTICULAR HYDROCARBONS FROM FOUR SAMPLE SIZES OF WORKERS FROM FIVE NESTS OF *Nasutitermes acajutlae* (HOLMGREN) FROM GUANA ISLAND, BVI, EXTRACTED ALIVE[a]

	25 workers		50 workers		100 workers		200 workers		
Hydrocarbon[b]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*F*
$_{23}$	1.33a	0.50	1.26ab	0.40	0.85bc	0.20	0.74c	0.14	10.82
$_{24}$	1.18a	0.59	0.99a	0.25	0.41b	0.18	0.41b	0.09	20.52
$_{25:1}$	0.00a	0.00	0.01a	0.05	0.33b	0.30	0.25b	0.13	15.31
$_{25}$	4.73a	1.31	4.05ab	1.15	2.84b	0.48	2.78b	0.44	15.66
$_{26}$	1.49a	0.53	1.01b	0.24	0.49c	0.18	0.45c	0.13	39.79
$_{27:1}$	0.14a	0.44	0.11a	0.21	0.42a	0.31	0.36a	0.15	4.03
$_{27}$	2.48a	1.23	1.97a	0.73	1.59a	0.65	1.44a	0.66	4.44
$_{28}$	1.17a	0.41	0.75b	0.19	0.32c	0.21	0.25c	0.10	41.75
$_{29}$	1.18a	0.39	0.73b	0.15	0.46bc	0.26	0.35c	0.10	32.58
$_{37:1}$	0.07a	0.18	0.14a	0.22	0.50b	0.18	0.48b	0.15	22.52
$_{38:1}$	0.21a	0.38	0.33ab	0.27	0.68bc	0.23	0.80c	0.16	15.89
$_{39:4}$	0.10a	0.22	0.15a	0.27	0.73b	0.34	0.81b	0.38	21.89
$_{39:2}$	0.00a	0.00	0.00a	0.00	0.00a	0.00	0.08a	0.16	3.70
$_{39:1}$	29.2a	2.47	28.7a	1.62	27.9a	2.70	26.4a	1.32	5.04
$_{40:1}$	2.01a	0.85	2.47ab	0.40	2.87b	0.13	3.23c	0.52	14.10
$_{41:4} + C_{41:5}$	5.47a	2.51	6.43a	2.44	7.92a	4.12	9.27a	2.52	4.70
$_{41:1}$	35.5a	2.79	32.2a	8.88	32.4a	2.90	30.4a	1.98	2.79
$_{42:1}$	0.99a	0.78	1.50ab	0.77	1.54ab	0.58	2.12b	0.66	6.50
$_{43:5}$	0.23a	0.50	0.94ab	0.97	1.49ab	1.44	2.20b	0.71	11.14
$_{43:2}$	0.00a	0.00	0.02a	0.08	0.03a	0.11	0.33b	0.11	49.69
$_{43:1}$	10.7a	1.26	11.5ab	0.87	12.5b	1.48	12.4b	1.11	7.10
$_{45:1}$	1.29a	1.10	1.96ab	0.84	3.21c	0.59	2.70bc	0.98	13.18

Means are from three samples from each of five separate nests ($N = 15$). Means for a given hydrocarbon followed by the same letter are not significantly different at the $\alpha = 0.00227$ level.

This shorthand (X-MeC_{XX} and $C_{XX:Y}$) uses descriptors for the location of the methyl group (X-Me), the total number of carbons (XX), excluding the methyl branch(es), and the number of double bonds (Y). Locations of double bonds are undetermined.

ples of 100 workers in seven cases and from samples of 200 workers in 11 cases. Samples of 100 and 200 workers yielded extracts with hydrocarbon proportions significantly different in only two cases. If workers were extracted while alive, it appears that the cuticular hydrocarbon mixtures change the least in samples of 100 or more.

Statistically significant differences ($\alpha = 0.00227$) in the percentage of hydrocarbon components among sample sizes were found for 13 hydrocarbon peaks when workers were extracted after drying (Table 5). Contrary to the results from live workers, the most abundant hydrocarbons $C_{39:1}$ and $C_{41:1}$, did display statistically significant differences among sample sizes. Samples of 25 workers yielded no statistically significant differences from samples of 50 workers for any hydrocarbons. Samples of 25 workers yielded extracts with hydrocarbon proportions significantly different from samples of 100 in 12 cases and from 200 workers in only five cases. Samples of 50 workers yielded extracts with hydrocarbon proportions significantly different from samples of 100 or 200 workers in only one case each (Table 5). Samples of 100 and 200 workers were not found to be significantly different for any of the 22 hydrocarbons analyzed. Similar to the result of extracting live workers, extracting workers after drying changed the least in sample sizes of 100 or more; however, extracts of samples of 50 workers were significantly different only for $C_{39:2}$, which had a relative abundance of 0.0% in samples of 25 or 50 workers and ca. 0.7% in samples of 100 or 200 workers.

The less abundant or trace compounds from extractions of the standard group size (100) are either missing or infrequently recorded (resulting in a lower mean value) in samples of 25 or 50 workers (Tables 4 and 5; Figures 6 and 7). The most abundant compounds, such as $C_{39:1}$ and $C_{41:1}$, have a lower mean value in samples of 100 or 200 workers than in groups of 25 workers (Tables 4 and 5; Figures 6 and 7). This undoubtedly results from the greater contribution of the minor compounds to the total hydrocarbon mixtures in the larger sample sizes; many of these trace compounds are not recorded in the samples of 25 or 50 workers, and they do not add to the total hydrocarbons (Figures 6 and 7). Most of the less abundant components fall below the line of equality (dashed line).

Thus, for workers of *N. acajutlae*, 100 appears to be the optimum sample size for adequately characterizing the cuticular hydrocarbons for quantitative comparisons using our operating parameters, regardless of whether the workers are extracted while alive or after drying. If the goal is to characterize all hydrocarbon components, even though they might be present in trivial amounts, then a larger sample (200–300 workers) should be taken, the extracts should be concentrated beyond our standard 60 μl, or the split ratio of the gas chromatograph should be altered. Collecting fewer insects in the field would save time and add to the convenience of field workers, but altering the chromatographic

TABLE 5. RELATIVE ABUNDANCE (MEAN PERCENT AND STANDARD DEVIATION) OF CUTICULAR HYDROCARBONS FROM FOUR SAMPLE SIZES OF WORKERS FROM FIVE NESTS OF *Nasutitermes acajutlae* (HOLMGREN) FROM GUANA ISLAND, BVI, EXTRACTED AFTER DRYING[a]

	25 workers		50 workers		100 workers		200 workers		
Hydrocarbon[b]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*F*
23	1.20a	0.38	1.08a	0.28	0.93a	0.20	1.12a	0.26	2.36
24	0.34a	0.34	0.39a	0.28	0.24a	0.23	0.36a	0.20	0.89
25 1	0.24a	0.41	0.40ab	0.27	0.64b	0.21	0.59ab	0.19	6.29
25	5.25a	2.44	3.83ab	1.09	3.04b	0.77	3.69ab	0.81	6.20
26	0.41a	0.35	0.41a	0.31	0.35a	0.29	0.41a	0.26	0.14
27 1	0.33a	0.43	0.58ab	0.22	0.89b	0.56	0.74ab	0.25	5.71
27	2.75a	1.62	2.49a	1.47	2.13a	1.44	2.65a	1.46	0.49
28	0.05a	0.13	0.25a	0.20	0.26a	0.22	0.29a	0.17	5.55
29	0.62a	0.50	0.69a	0.42	0.84a	0.52	0.85a	0.50	0.81
37 1	0.12a	0.22	0.22ab	0.15	0.47b	0.07	0.43ab	0.15	16.98
38 1	0.26a	0.35	0.45ab	0.16	0.57b	0.10	0.63b	0.21	8.03
39 4	0.97a	0.75	1.30ab	0.43	2.00b	0.68	1.91ab	0.92	7.07
39 2	0.00a	0.00	0.00a	0.00	0.67b	0.46	0.69b	0.28	31.89
39 1	22.6a	1.99	21.3ab	1.57	19.6b	1.97	19.6b	1.79	9.23
40 1	1.90a	0.29	2.05ab	0.33	2.41b	0.19	2.27ab	0.55	5.85
41 4 + $C_{41\ 5}$	18.6a	4.60	18.9a	3.05	19.4a	3.39	17.4a	2.43	0.87
41 1	28.7a	2.20	26.3ab	1.85	23.1b	1.77	23.4b	3.16	19.09
42 1	1.33a	1.02	1.75a	0.80	1.72a	0.52	1.44a	0.49	1.19
43 5	3.10a	1.58	4.17a	0.98	4.25a	1.92	4.40a	1.09	2.54
43 2	0.12a	0.47	0.48ab	0.32	0.91b	0.63	0.94b	0.37	10.62
43 1	9.58a	1.01	9.84a	0.76	10.0a	1.77	10.3a	1.30	0.78
45 1	1.35a	0.72	1.89ab	0.54	2.47b	0.55	2.26ab	0.83	7.99

Means are from three subsamples from each of five separate nests ($N = 15$). Means for a given hydrocarbon followed by the same letter are not significantly different at the $\alpha = 0.00227$ level.

This shorthand (X-MeC_{XX} and $C_{XX\ Y}$) uses descriptors for the location of the methyl group (X-Me), the total number of carbons (XX), excluding the methyl branch(es), and the number of double bonds (Y). Locations of double bonds are undetermined.

The *F* statistic has a $P < 0.00227$, yet the Tukey's procedure did not separate any of the means for this hydrocarbon.

parameters would increase the complexity and repeatability of laboratory operations. If a sample of 100 workers is not attainable, then concentration of the extract or altering the split ratio must be done or the sample will not be usable.

It is likely that our earlier problem with quantifying hydrocarbons from *N. costalis* and *N. ephratae* (Haverty et al., 1990b) resulted from two confounding problems. First, the workers were extracted live, in the field, which resulted in a less efficient extraction of the hydrocarbons, especially the less abundant components. Second, while 100 workers were used in the extractions, due to the

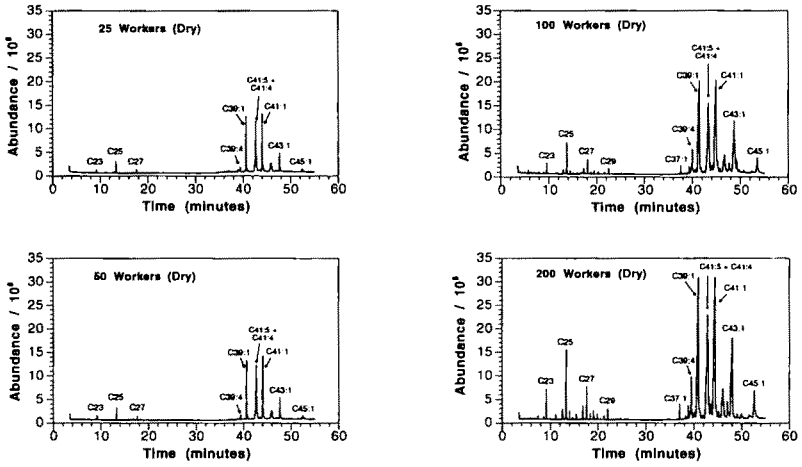

FIG. 6. Chromatograms of cuticular hydrocarbons extracted with 10 ml hexane for 10 min from four sample sizes (25, 50, 100, and 200) of dried workers from one colony of *Nasutitermes acajutlae* from Guana Island, BVI.

size of *N. ephratae*, these collections could very well have been below an acceptable size. Large workers of *N. ephratae*, (dry weight = 0.6 mg) are considerably smaller than large workers of *N. acajutlae* (dry weight = 1.3 mg) (Thorne, 1985); 100 large workers of the former species may not have had sufficient surface lipids to provide an adequate chromatogram.

Effects of Solvent

For field entomologists working in the tropics it would be convenient if storage of termites in 100% ethanol (EtOH) allowed for equivalent extraction of hydrocarbons. Preliminary observations of S. J. Seybold and L. J. Nelson (personal communication) indicated that EtOH will extract cuticular hydrocarbons of adult *Ips pini* (Say) (Coleoptera: Scolytidae). Furthermore, the longer beetles were in EtOH, the greater the extraction efficiency. Their work showed: (1) that the EtOH-extracted insects can be further extracted with hexane, (2) that the EtOH extract can be dried and the lipids redissolved in hexane, and (3) that the hexane solutions of the EtOH extract and the EtOH-extracted-insect extract can be recombined to provide a reconstituted extract for characterization. They have not shown whether the resulting hydrocarbon mixture is comparable to an extract gathered by a standard 10-min extraction with hexane of live or dead insects.

Unfortunately, the resulting extracts for both workers and soldiers of *N.*

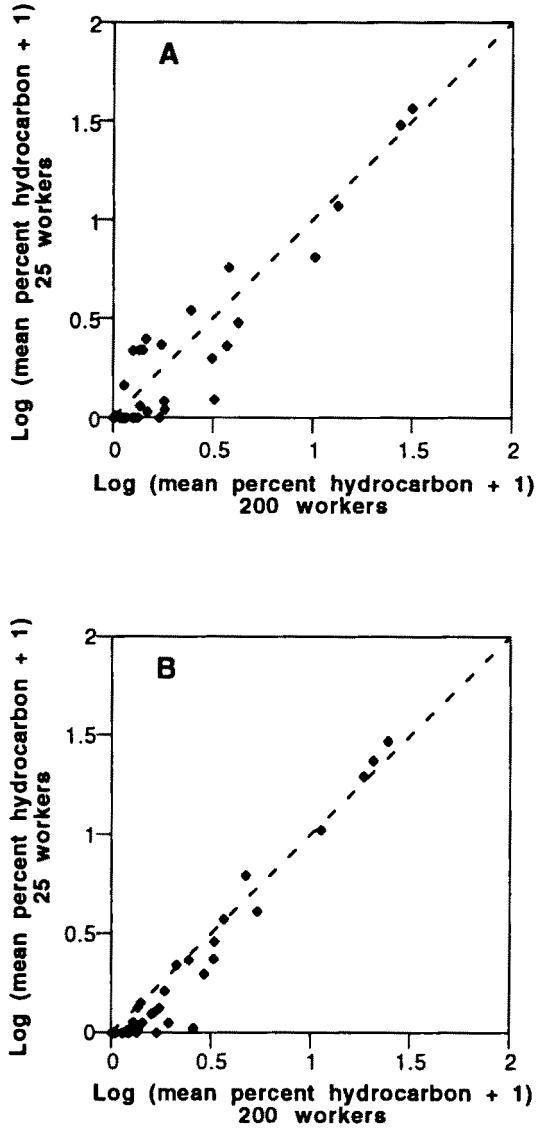

FIG. 7. Log (mean percent + 1) of 34 cuticular hydrocarbon components for groups of 25 and 200 workers extracted either alive (A) or after drying (B). Note: transformation was not done for statistical purposes, but to spread out the points for the less abundant components.

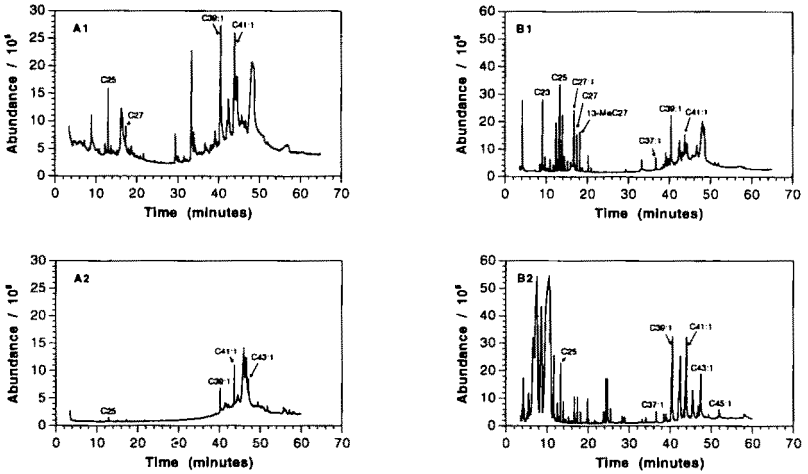

FIG. 8. Chromatograms of cuticular hydrocarbons (and other compounds) from 200 workers (A) or 200 soldiers (B) collected into 100% ethanol. For each caste the first chromatogram (A1 or B1) represents the pattern of compounds that were extracted by the ethanol. The second chromatogram for each caste (A2 or B2) represents the pattern of compounds extracted from the termites after they were stored in ethanol for 60 days, subsequently dried, then extracted with 10 ml of hexane for 10 min.

acajutlae stored in ethanol were not comparable to those where the standard technique was utilized. Storage in ethanol caused numerous, hexane-soluble, unidentified, nonhydrocarbon compounds to be extracted or these nonhydrocarbon peaks were in the ethanol as denaturing components (Figure 8). These compounds were not removed after evaporating the ethanol under nitrogen, redissolving in hexane, and pipetting through activated BioSil-A. Furthermore, enormous amounts of nitrogen and time were required to dry the ethanol samples. As a result of these problems, we did not characterize all 15 samples, because we presumed such a course would only waste resources and could affect our GC-MS. Therefore, unless a different cleanup procedure is developed, storage in ethanol is unacceptable for characterizing the hydrocarbons from *Nasutitermes*.

CONCLUSIONS

Different colonies of *N. acajutlae* produce qualitatively similar, but quantitatively dissimilar hydrocarbon mixtures. These colony-specific profiles appear to be reproducible; multiple extracts from separate samples of each colony are qualitatively identical and quantitatively quite similar. Only one extraction of a

sample of workers is necessary. A very brief extraction (in 10 ml of hexane for only 20 sec) of live workers produces a mixture of hydrocarbons equivalent to a 10-min or a 24-hr extraction. Holding a sample of workers (or soldiers) in hexane for a period of two years results in a radically different extract than the standard 10-min extraction and is not recommended.

Drying workers of *N. acajutlae* before extraction results in highly significant increases in the relative amounts of $C_{41:4}$ and $C_{41:5}$ and an apparent decrease in the relative amounts of the most abundant compounds, $C_{39:1}$, $C_{41:1}$, and $C_{43:1}$. In general, drying workers prior to extraction tends to enhance extraction of the less abundant unsaturated compounds and does not result in equivalent mixtures of hydrocarbons when compared to extraction of live workers. Extracting a minimum of 100 workers (live or dried) with hexane for 20 sec to 10 min seems to be the best method for characterizing cuticular hydrocarbons of *N. acajutlae*. For smaller species, samples of at least 200 would probably guarantee a satisfactory chromatogram.

For quantitative comparisons, the extraction technique should ideally be the same for all samples, i.e., hydrocarbon mixtures extracted from dried samples should not be compared to those extracted from live samples. Extraction of either live or dried termites would suffice for characterization of cuticular hydrocarbons for ecological studies but may not be comparable for taxonomic purposes. For quality of chromatograms and for several logistical reasons, we obtained the best results by drying at least 100 termites and then extracting them by the standard technique (10 min in 10 ml hexane) in the laboratory. Extraction of live termites in the field requires twice the number of vials and transportation of flammable liquids. Even with the potential logistical difficulties involved with drying termites in the tropics, we recommend this as the optimal technique to use.

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SELF-GERMINATION INHIBITORS FROM *Colletotrichum fragariae*

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Abstract—The conidial germination of *Colletotrichum fragariae* in water was dependent on population, size germination was inhibited at higher concentrations of conidia in water. The inhibition was considered to be caused by some germination-inhibiting substances exuded from conidia, since the germination did occur after washing the conidia repeatedly. The germination-inhibiting substances were searched for in the acetone extract of potato–sucrose–agar (PSA) cultures of the fungus and five active compounds were isolated. Their structures were characterized spectroscopically. HPLC analysis indicated that one of the isolated compounds among these was exuded not only from mycelia but also from the conidia in water under crowded conditions. We suggest that these compounds control the conidial germination of the fungi.

Key Words—Self-inhibitor, conidia, germination, anthracnose, TLC plate bioassay, *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*, *Glomerella fusarioides*.

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INTRODUCTION

Spore germination is inhibited under crowded conditions in a number of fungi over a wide range of taxonomic groups. This phenomenon is considered to be due to fungal metabolite(s) called self-inhibitors that are secreted from the spores (Allen, 1976). These may delay germination until the conditions become more advantageous for fungal growth by spore dissemination (Gottleib, 1973; Allen, 1976; Lax et al., 1985).

The genus *Colletotrichum* or *Glomerella* (anthracnose fungus) is among diverse genera that have been studied with respect to self-inhibition. The existence of a self-inhibitor in this genus was first predicted by Lingappa and Lingappa (1965), who detected several substances with germination-inhibiting activity in extracts of the conidia and mycelia of *Glomerella cingulata* (the perfect stage of *Colletotrichum gloeosporioides*), but failed to isolate and identify them (Lingappa and Lingappa, 1966, 1967, 1969; Lingappa et al., 1973). Lax et al. (1985) successfully isolated the self-inhibitor from *C. gloeosporioides* f. sp. *jussiaea* and identified it as gloeosporone (Meyer et al., 1983), although the proposed structure was revised by later chemical studies (Meyer et al., 1987). Tsurushima et al. (1995) also examined self-inhibitors of this fungus. Although they could not detect a gloeosporone, they did detect three active compounds in the conidial suspension as well as in the culture extract. These compounds inhibited conidial germination and were identified as (*E*)- and (*Z*)-3-ethylidene-1,3-dihydroindol-2-one and (2*R*)-(3-indolyl) propionic acid, respectively. In *C. graminicola*, Leite and Nicholson (1992, 1993) found that the self-inhibitory activity was attributed to the presence of mycosporine–alanine and volatile metabolites.

In this paper, we report that the germination of *C. fragariae* is dependent on the population and is controlled by self-inhibitors.

METHODS AND MATERIALS

Fungi and Culture Conditions

Isolates of *Colletotrichum fragariae* Brooks, *C. gloeosporioides* f. sp. *jussiaea* Peens., *C. gloeosporioides* f. sp. *aeschynomene* Penz., *C. gloeosporioides* (isolate no. 7696), and *C. graminicola* Wilson were provided by Dr. G. Templeton, University of Arkansas. *C. lindemuthianum* Briosi et Cavara IFO 5260, *Glomerella cingulata* Spaulding et Schrenk IFO 7498, *G. furctigena* Saccardo IFO 5951, *G. gossypii* Edgerton IFO 6181, and *G. fusarioides* Edgerton IFO 8831 were obtained from the Institute of Fermentation, Osaka. An isolate of *C. lagenarium* Ellis et Halsted was provided by Dr. T. Tani of Kagawa University. Each strain was cultured and maintained at 26°C in the dark in potato–sucrose–

agar (PSA) medium composed of potato (200 g), sucrose (20 g), agar (30 g) and water (1 liter).

Conidia for the bioassay and for the analysis of inhibitor were harvested by gently brushing 7-day-old PSA plate cultures after adding about 6 ml distilled water. This was filtered through three layers of gauze, and centrifuged at 2350 *g* for 10 min. The conidial pellet was washed twice by resuspension and centrifuged in distilled water before use.

Self-Inhibition Studies

Density Effects. To investigate the effect of densities on germination, conidial suspensions (50 μl) at concentrations of 10^3–10^7/ml were placed in hole slide glass plates, and incubated at 26°C for 20 hr in a moist chamber. The rate of germination was determined microscopically. Three hundred conidia from random fields were counted per plate.

Exudate Effects. The effect of conidia exudates on germination was also examined. The hole slide glass plates were inoculated with 50 μl of a suspension containing 2×10^6 conidia/ml. The water in the suspension was replaced hourly by elimination with filter paper, and the conidia were resuspended with 50 μl of sterile deionized water. A suspension without water replacement was incubated as a control. The conidia were incubated and counted as before.

Purified Inhibitors. Fresh conidia of *C. fragariae* were inoculated in an aqueous solution of a known concentration of a test sample on hole slide glass plates, and the density was adjusted to 10^5 conidia/ml. An aqueous solution containing 0.5% methanol served as control. Inoculated plates were incubated at 26°C for 20 hr under moist conditions, and the percentage of germinated conidia was determined microscopically.

Isolation of Germination Self-Inhibitory Compounds

Germination-inhibitory compounds were isolated from the PSA cultures of *C. fragariae* as shown in Figure 1. Two-week-old cultures were extracted with acetone, and the extract was concentrated under reduced pressure to obtain the residue. This residue was then extracted with ethyl acetate and dried. The dried extract was loaded on a silica gel column, and the column was eluted with a *n*-hexane–ethyl acetate–methanol gradient.

During these separation procedures, the germination-inhibitory compounds were monitored by TLC plate bioassay using *C. lagenarium* as the test fungus (Ueno et al., 1991; Tsurushima et al., 1995). Samples were applied to silica gel TLC plates, and the developed plates were sprayed with PSA medium containing the conidia of *C. lagenarium* for incubation at 26°C in a moist chamber. Inhibitory compounds were recognized as colorless spots in the black-colored

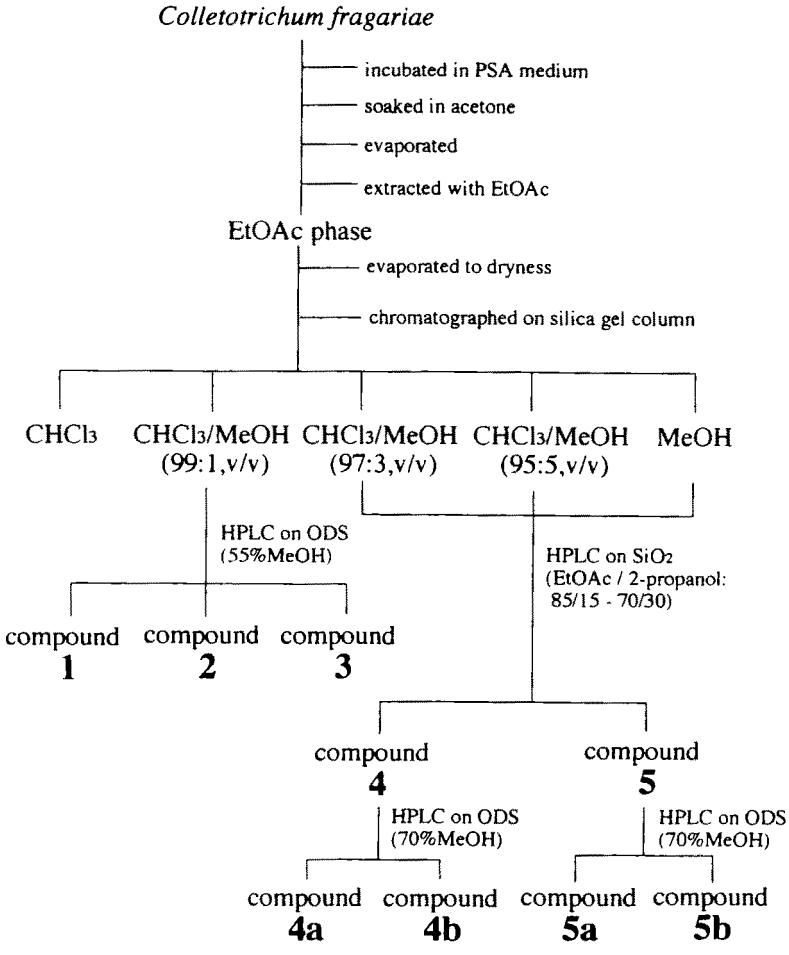


FIG. 1. Isolation of self-inhibitors from culture of *Colletotrichum fragariae*.

background as the fungus grew. A solvent system of benzene–EtOAc–MeOH (50:50:1, v/v) was used for TLC.

Active compounds detected in the $CHCl_3$–MeOH (99:1) fraction were purified by reverse-phase HPLC to give compounds **1–3** (Figure 4). The isolated compounds were identified by spectroscopic analyses. The physicochemical data were as follows:

(*E*)-3-ethylidene-1,3-dihydroindol-2-one (**1**): MS (EI 70eV) *m/z* (%);

159.0643(M^+, 100%, $C_{10}H_9N_1O_1$), 144(52%), 130(42%), 103(5%). ^{1}H NMR (400 MHz, $CDCl_3$) δ; 2.29(3H, d), 6.91(1H, d), 7.03(1H, t), 7.12(1H, q), 7.21(1H, t), 7.57(1H, d), 8.70(1H, s). IR(KBr); 1715 cm^{-1}($\nu_{C=O}$). [α] 0 ($CHCl_3$).

(*Z*)-3-ethylidene-1,3-dihydroindol-2-one (**2**): MS (EI 70eV) *m/z* (%); 159(M^+, 100%), 144(63%), 130(53%), 103(7%). ^{1}H NMR(400 MHz, $CDCl_3$) δ; 2.46(3H, d), 6.83(1H, d), 6.97(1H, q), 6.99(1H, t), 7.18(1H, t), 7.37(1H, d), 7.95(1H, s). IR(KBr); 1712 cm^{-1} ($\nu_{C=O}$). [α] 0 ($CHCl_3$).

(2*R*)-(3-indolyl) propionic acid (**3**): MS (EI 70eV) *m/z* (%): 190 ($[M+H]^+$, 32%), 189(M^+, 23%), 175(3%), 144(100%), 117(9%), 115(9%). ^{1}H NMR (400 MHz, CD_3OD) δ; 1.57(3H, d), 3.97(1H, q), 7.00(1H, d), 7.08(1H, t), 7.15(1H, s), 7.33(1H, d), 7.66(1H, d). [α] -60 (CH_3OH).

Active compounds in the $CHCl_3$–MeOH (97:3 and 95:5) and 100% MeOH fractions were purified by preparative HPLC (UV 254 nm, Cosmosil 10SL 20 × 250 mm column, *n*-hexane–2-propanol gradient of 85:15, 0–22 min; 70:30, 22–50 min, 5 ml/min) to give compounds **4** and **5**.

Analyses of Isolated Inhibitors. The presence of isolated inhibitors in a *C. fragariae* conidia suspension or in another anthracnose fungal culture was analyzed by HPLC. Conidia of *C. fragariae* were suspended in 20 ml of water, with the density being adjusted to 10^7 conidia/ml, and incubated in a capped test tube for 16 hr in a rotary shaker. The suspension was then centrifuged at 2350*g* for 10 min. The supernatant was extracted twice with an equal volume of ethyl acetate. A 2-week-old fungal culture on PSA medium was extracted with acetone. The acetone extract was evaporated under reduced pressure, and the aqueous fraction remaining was then extracted with ethyl acetate. These ethyl acetate extracts were concentrated and subjected to the HPLC analysis on a Cosmosil 5C18AR column and eluted with a mobile phase of 70% MeOH in water. The compounds were identified on the basis of the retention-time on HPLC. The identity was also confirmed by LC-MS measured with a Hitachi M-1000 spectrometer operated in positive ion mode.

RESULTS

Effect of Conidial Concentration on Germination

The germination of conidia of *C. fragariae* was inversely dependent on the conidial concentration (Figure 2). The rate of germination at 10^4 conidia/ml exceeded 80%, whereas the density at 10^7 conidia/ml was less than 10%. Inhibition of the germination at high conidial concentration was reduced by washing the conidia to remove the exudates (Figure 3).

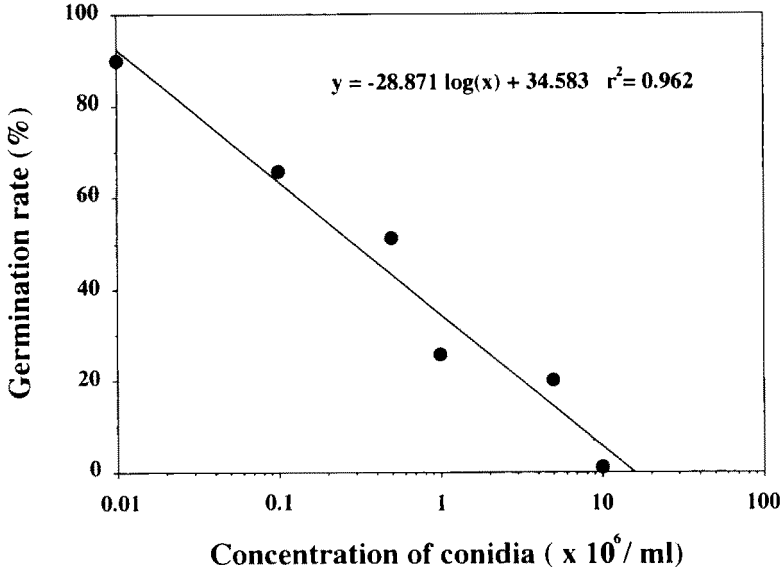


FIG. 2. Relationship between germination rate and *Colletotrichum fragariae* conidial densities.

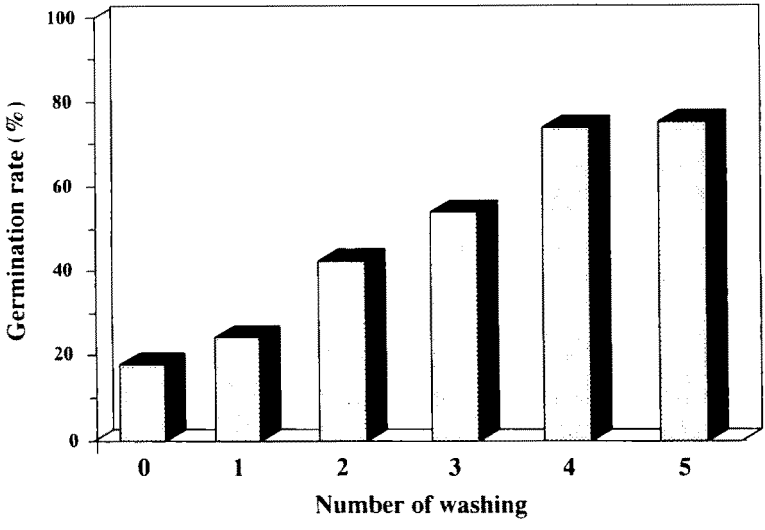
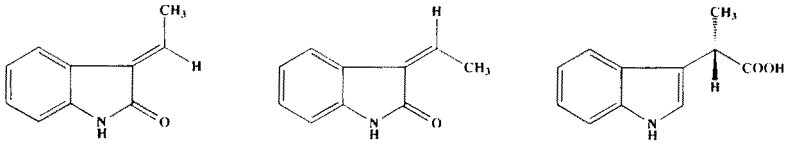


FIG. 3. The effect of washing on conidial germination.



Compound **1** Compound **2** Compound **3**

FIG. 4. Structures of the self-inhibitors from culture of *Colletotrichum fragariae*.

Isolation of Self-Inhibitors from C. fragariae

Germination-inhibitory compounds were searched for in the acetone extract of *C. fragariae* cultured on PSA plates, and active compounds **1–5** were isolated.

Compounds **1–3** were identified as (*E*)- and (*Z*)-3-ethylidene-1,3-dihydroindol-2-one and (2*R*)-(3-indolyl) propionic acid, respectively, on the basis of their physicochemical data (Figure 4). These have also been described as self-inhibitors CG-SI 1, 2, and 3, respectively, from *C. gloeosporioides* (Tsurushima et al., 1995).

Although active compounds **4** and **5** purified by preparative HPLC following silica gel column chromatography gave single spots on TLC in three solvent systems, compound **4** consisted of the compounds **4a** and **4b**, and compound **5**, consisted of **5a**, **5b** according to reverse-phase HPLC (Figure 5). These four compounds gave the essentially same LC-mass spectra with $M+H^+$ ions at *m/z* 387, suggesting that they were isomers.

Analysis of Isolated Inhibitors in Conidia Suspension Solutions

In order to examine whether the isolated germination-inhibitory compounds are actually secreted from conidia, the ethyl acetate extracts of a conidia suspension of *C. fragariae* were analyzed by HPLC and LC-MS. Four compounds detected on the chromatogram (Figure 6) were identical with **1**, **2**, **4a**, and **4b** in terms of chromatographic behavior and MS. Additionally, two unknown compounds were detected on HPLC (peaks c and d in Figure 6), the molecular weights of which were estimated to be the same (386) as those of **4a**, **4b**, **5a**, and **5b** according to LC-MS analyses.

Germination-Inhibitory Activity of Isolated Compounds

Compounds **1–5** inhibited the conidial germination of *C. fragariae* with ED_{50} values of 3, 5, 25, 20, and 15 μg/ml, respectively.

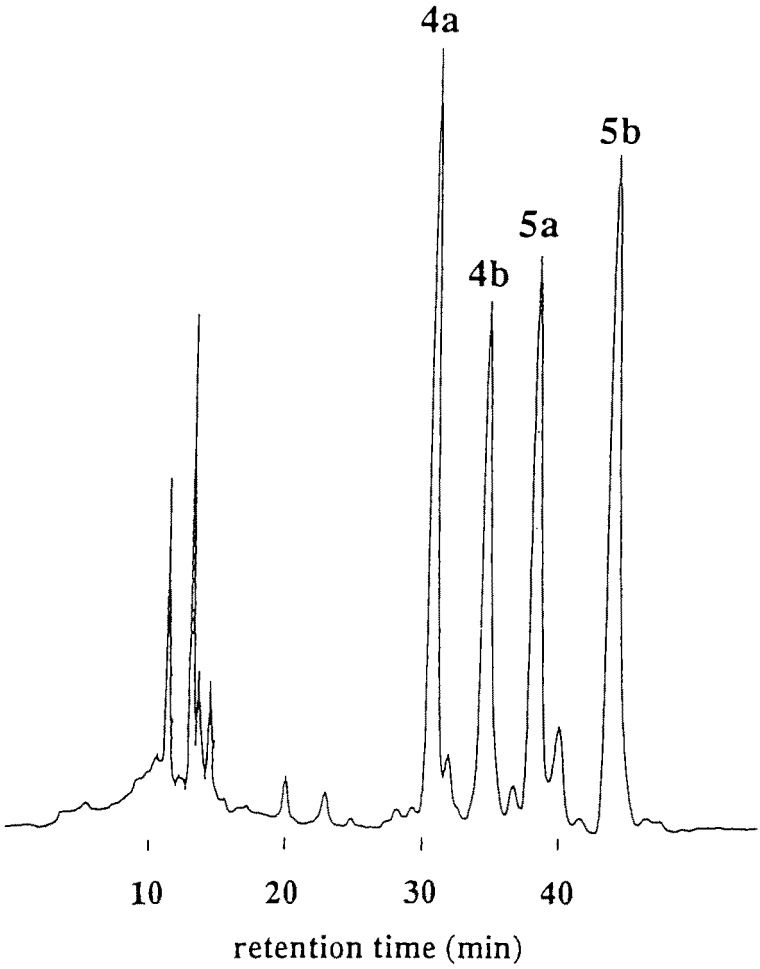


FIG. 5. HPLC chromatogram of self-inhibitor compounds **4** and **5**, from culture of *Colletotrichum fragariae*. Analytical conditions: column—Cosmosil 5C18AR 10 × 250 mm; mobile phase—70% MeOH; flow rate—2 ml/min; detection—UV absorption at 254 nm.

Distribution of Compounds 4 and 5 in Various Species of Anthracnose Fungi

The distribution of new compounds **4** and **5** was surveyed in various species of the genera *Colletotrichum* and *Glomerella* by HPLC and LC-MS. As shown in Table 1, compounds **4** and **5** were present only in a single species, *Glomeralla fusarioides*, in addition to *C. fragariae*.

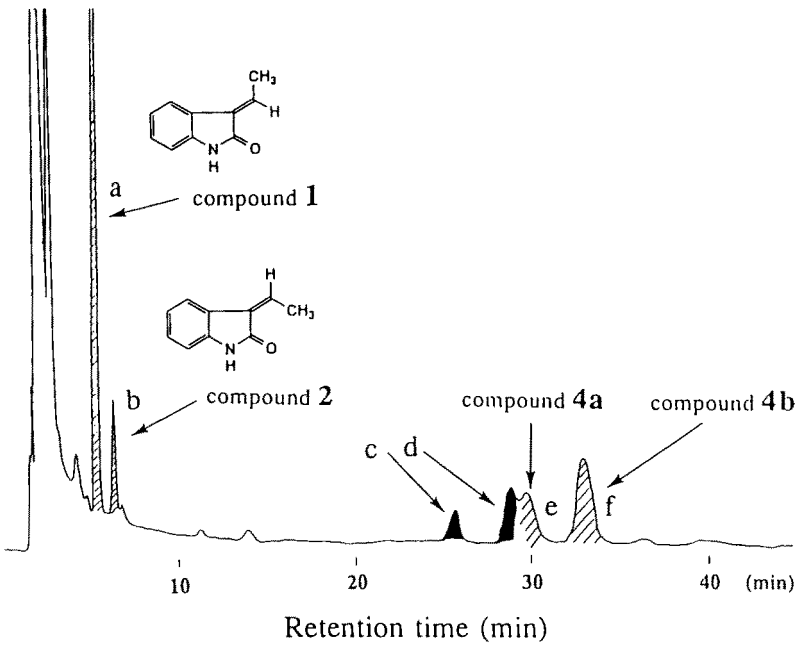


FIG. 6. Detection of self-inhibitors in a suspension of *Colletotrichum fragariae* conidia. Analytical conditions: column—YMC ODS A-302 4.6 × 150 mm; mobile phase—55% MeOH; flow rate—1 ml/min; detection—UV absorption at 254 nm.

DISCUSSION

This is the first paper to describe self-germination inhibition in *C. fragariae* and to demonstrate that conidial germination is inhibited in water at high spore density. A similar phenomenon previously has been observed in other anthracnose fungi, and the germination inhibition was putatively attributed to a "self-inhibitor" that is exuded from the conidia into the germinating fluid (Richardson and Thorn, 1962; Lingappa and Lingappa, 1965, 1966, 1967, 1969; Lingappa et al., 1973). This study has shown that such an endogenous self-inhibitor(s) from conidia is also present in *C. fragariae* and is possibly involved in the observed population-dependent germination inhibition, as the inhibition was diminished by washing the conidia repeatedly.

We searched for the germination-inhibitory compounds by directly extracting the whole culture of *C. fragariae* with organic solvents, and after chromatographic separation of the extract, we isolated active compounds **1**–**5**. Compounds **1**–**3** are indole derivatives, which are identical with the self-inhibitors from *C. gloeosporioides* (Tsurushima et al. 1995). Although the presence of

TABLE 1. DETECTION OF SELF-INHIBITORS IN CULTURE OF SOME ANTHRACNOSE FUNGI

Anthracnose fungi	Compound				
	1	2	3	4	5
Colletotrichum gloeosporioides f.sp. *jussiaea*	+[a]	+	+	−	−
C. gloeosporioides f.sp. *aeschynomene*	−	−	−	−	−
C. gloeosporioides isolate 7696	−	−	−	−	−
C. graminicola	−	−	−	−	−
C. fragariae	+	+	+	+	+
C. lagenarium	−	−	−	−	−
C. lindemuthianum IFO 5260	−	−	−	−	−
Glomerella cingulata IFO 7498	−	−	−	−	−
G. fructigene IFO 5951	−	−	−	−	−
G. gossypii IFO 6181	−	−	−	−	−
G. fusarioides IFO 8831	−	−	−	+	+

[a] +: detected, −: not detected.

these compounds in the culture of *C. fragariae* was predicted in a previous study on the basis of HPLC analysis (Tsurushima et al, 1995), this study has confirmed it by spectroscopic analysis of the isolated compounds. Compounds **4** and **5** could not be fully characterized in this study, but the available data suggested that they are novel, at least as self-inhibitors. Reverse-phase HPLC analyses indicated that both of them consisted of pairs of isomers, the properties of which are discussed in more detail below.

Compounds **1**, **2**, **4a**, and **4b** were also detected in the conidial suspension of *C. fragariae*. This result indicates that these compounds are not artifacts arising from solvent extraction of the culture, as contended by Allen (1976), but are genuine factors that are exuded from conidia and play important roles in the regulation of germination. Although compounds **3** and **5** were not detected in the conidial suspension examined in this study, they appeared to be unstable and may have possibly undergone decomposition during the analytical procedures if their amount was small. In this context, **3** was observed to change into **1** and **2** with exposure to light. Moreover, unknown compounds in the conidial suspension (corresponding to peaks a and d in Figure 6) have the same molecular weight as **5a** and **5b**, suggesting that they could have been produced by isomerization of **5a** and **5b**. Because the self-inhibitory activities of these unknown compounds were not determined here, their roles and significance remain unclear.

The inhibitory activities of **1** and **2** were about five times more potent than those of **4** or **5**. These findings, as well as the apparent abundance of **1** and **2** in the conidial suspension as shown in Figure 6, suggest that these compounds contribute mainly to the germination self-inhibition, although the compounds may act synergistically.

The germination inhibitory activities of **1** and **2** have been extensively examined by Tsurushima et al. (1995), who found that **1** and **2** were inhibitory only against the species that produced them, *i.e.*, *C. gloeosporioides* f. sp. *jussiaea* and *C. fragariae*, among 16 species of anthracnose fungi. In this study, the distributions of **4** and **5** were similarly surveyed among anthracnose fungi. We detected them only in *G. fusarioides* and *C. fragariae*. The species-specificity of the inhibitory activities of **4** and **5** is currently under investigation.

Compounds **4** and **5** were so unstable that structural studies were frequently hampered by decomposition during the isolation procedures. These compounds consisted of pairs of isomers according to HPLC analyses. Among these isomers, the relatively stable **4b** could be isolated by rapid and careful manipulation and preparative HPLC. Structural studies by IR, UV, MS, and NMR will be reported in detail elsewhere.

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Glomerella species. We wish to thank Dr. Keiji Hashimoto, Kyoto Pharmaceutical University, for LC-MS measurements. This work was financially supported by the Mitsubishi Foundation.

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A COMPARISON OF THE EFFECTIVENESS OF PREDATOR ODOR AND PLANT ANTIFEEDANT IN DETERRING SMALL MAMMAL FEEDING DAMAGE ON LODGEPOLE PINE SEEDLINGS

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Abstract—The influence of mustelid (weasel) anal-gland compounds (semiochemicals) and a plant antifeedant (pinosylvin) on snowshoe hare (*Lepus americanus*) and meadow vole (*Microtus pennsylvanicus*) feeding damage was investigated. This study has shown conclusively that when applied to spring-planted lodgepole pine seedlings, pinosylvin is effective in significantly reducing feeding damage. Seedlings treated with pinosylvin suffered damage levels of 2.3% and 21.2% compared to the controls with damage levels of 24.5% and 41.0%. Weasel scent was found to reduce damage levels, but the reduction was not statistically significant in all blocks.

Key Words—Synthetic weasel odor, semiochemical, plant antifeedants, pinosylvin, vole, snowshoe hare, biological control, *Microtus pennsylvanicus*, *Lepus americanus*.

INTRODUCTION

Both snowshoe hares (*Lepus americanus*) and meadow voles (*Microtus pennsylvanicus*) are widely distributed across much of boreal North America. Both the hare and vole are known to undergo cyclic fluctuations in abundance, the snowshoe hare having an 8- to 11-year cycle (Keith and Windberg, 1978; Keith et al., 1984; Krebs et al., 1986) and the meadow vole having a 3- to 5-year

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cycle (Krebs et al., 1969). During the years of peak hare or vole numbers, feeding damage to conifer plantations by either or both species can be a serious problem (Radvanyi, 1987; Bergeron and Tardiff, 1988; Sullivan and Sullivan, 1983; Sullivan et al., 1988a, 1992).

Both snowshoe hares and meadow voles damage conifer seedlings by clipping the terminal and lateral shoot or feeding upon the cambium of the stem. In all cases the damage inflicted by these herbivores can have serious and devastating consequences for the conifer plantation. In the past, toxicants have been used, unsucessfully, in an attempt to control these pest species. More recently the development of synthetic plant antifeedant compounds and predator odors has provided a more successful alternative for dealing with hare and vole feeding damage.

Pinosylvin. Under natural conditions browsing by snowshoe hares on a variety of boreal plant species results in the production of adventitious shoots that are unpalatable to snowshoe hares (Bryant and Kuropat, 1980). The likely reason for this avoidance of the adventitious shoots is the production of significantly higher concentrations of terpene and phenolic resins in the adventitious shoots than is found in the mature growth forms of the same species (Bryant, 1981).

Pinosylvin, a naturally occurring plant phytoalexin, has been found to be produced in high concentrations by the adventitious shoots of Alaskan green alder (*Alnus crispa*). Laboratory bioassays have determined that this compound deters snowshoe hare feeding and probably explains the avoidance of the juvenile shoots in the field (Bryant et al., 1983; Clausen et al., 1986).

Sullivan et al. (1992) provided both pen and field trials demonstrating that pinosylvin significantly reduced snowshoe hare feeding on lodgepole pine seedlings. This biological control method takes advantage of the herbivores' recognition of an unpalatable substance.

Semiochemicals. Certain predator odors originating from feces, urine, or scent (anal) gland secretions elicit an innate fear response when detected by prey species (Gorman, 1984; Kavaliers, 1988; Jedrzejewski and Jedrzejewska, 1990). This response can result in increased vigilance, less feeding, reduced reproduction, or avoidance of the area (Stoddart, 1976, 1980; Ylönen, 1989, Jedrzejewski and Jedrzejewska, 1990). To date, natural and synthetic predator odors have produced significant avoidance responses in several pest species and reduced damage to forest and agricultural crops (Sullivan and Crump, 1984; Sullivan et al., 1985, 1988b, 1990).

There is also evidence that chemical information exchange occurs within predator species through the secretion of certain compounds. Synthetic anal-gland secretions of the ferret (*Mustela furo*) and urine of the red fox (*Vulpes vulpes*) act as signaling pheromones that attract conspecifics (Whitten et al., 1980; Clapperton et al., 1988, 1989). Zimmerling and Sullivan (1994) presented

evidence that a synthetic mustelid anal-gland compound attracted weasels to treated sites.

The attraction of weasels to a site should increase predation on the prey species, thereby reducing numbers and reinforcing the innate fear response. Thus use of predator odors in combination with plant phytoalexins results in biological control that takes advantage of both the innate fear response of the prey animals and the attraction of predators.

This paper reports on the comparative effectiveness of pinosylvin and synthetic weasel odor in deterring feeding damage by snowshoe hares and meadow voles on lodgepole pine seedlings.

METHODS AND MATERIALS

Study Area. This study was undertaken in the fall of 1994 in Tree Farm License #1 of Skeena Cellulose Inc. near the city of Terrace, British Columbia, Canada. The areas chosen were suffering high levels of feeding damage as voles were in the peak of their population cycle and hare numbers were rising. Two blocks served as replicates to determine the effectiveness of weasel scent and pinosylvin at deterring feeding damage on spring-planted lodgepole pine plug stock. The blocks were 556100 (block A), a 10-ha trial area in an 88-ha cutblock, planted with lodgepole pine in the spring of 1994, and 312900E (block B), a 6.7-ha trial area in a 24-ha cutblock, planted with lodgepole pine in the spring of 1994.

Field Procedures. Within each study site, three 1- to 2-ha sections were marked. Within one of these sections, 200 randomly chosen seedlings were fitted with weasel scent release devices attached to the terminal or upper lateral with a pipe cleaner. The formulation of mustelid semiochemicals was produced by Phero Tech Inc. Delta, British Columbia, Canada. The compounds were encapsulated in 6-cm × 0.4-cm composite polymeric cylinders. These devices consist of a 0.5-mm-thick sheath and a core plastisol formulated with 10 mg active ingredient. This type of device was determined to yield the best controlled release of the odor (Sullivan et al., 1990). The active ingredient was a 1:1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane after Crump (1978, 1980, 1982).

A second section had 200 randomly chosen seedlings fitted with a plant antifeedant device containing pinosylvin attached to the terminal or upper lateral with a thin wire. The formulation of pinosylvin was produced by Phero Tech Inc. Delta, British Columbia, Canada. Pinosylvin (3,5-dihydroxy stilbene) was encapsulated in a 3.4-cm × 0.3-cm monolithic release device. These devices consist of a sheath and a core plastisol formulated with 32 mg active ingredient. The third section had 200 randomly chosen control seedlings.

Each treated and control seedling was identified in the fall by a blue-painted stake. In April 1995, all treated and control seedlings were revisited and the presence of feeding damage by snowshoe hares or voles was recorded. Damage was recorded as clipped terminal, clipped lateral, both terminal and lateral clipped, or stem gnawed.

RESULTS

Figure 1 gives a comparison of feeding damage within blocks A and B. In both blocks, pinosylvin treated seedlings had significantly lower levels of feeding damage as compared to the control (χ^2, $P < 0.05$). The pinosylvin-treated areas suffered 2.3% damage in block A, and 21.2% in block B, compared to the control areas, which had damage levels of 24.5% and 41.0% in blocks A and B, respectively. In block A, damage to seedlings receiving weasel scent (10.1%) was also significantly different from damage in the control area (χ^2, $P < 0.05$); however, in block B, damage in weasel-scent-treated areas (31.5%) was not significantly different from unprotected seedlings in the control area (χ^2, $P > 0.05$).

In block A, rates of damage were not significantly different between the weasel-scent-treated area (10.1%) and the pinosylvin-treated area (2.3%) (χ^2,

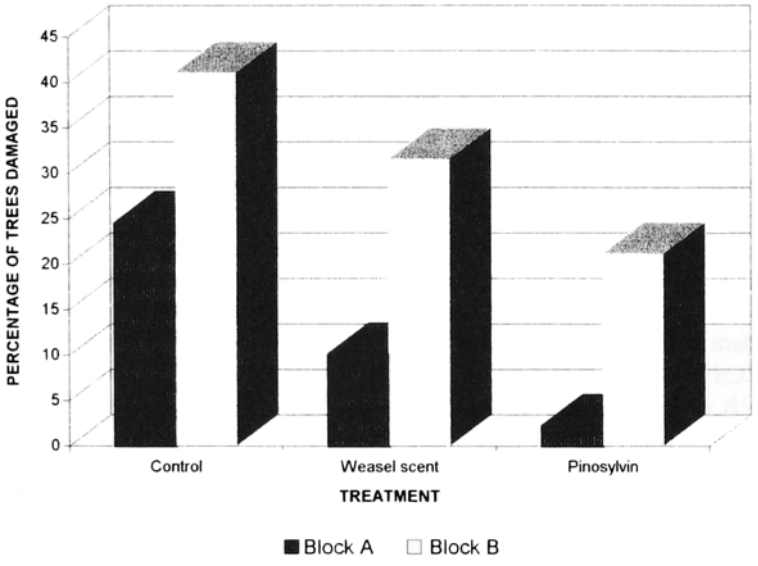

FIG. 1. Feeding damage in blocks A and B.

$P > 0.05$). In block B, the amount of damage in the weasel-scent-treated area (31.5%) was significantly different (χ^2, $P < 0.05$) from damage in the pinosylvin-treated area (21.2%).

Figures 2 and 3 give a breakdown of the type of damage inflicted. In both blocks, the majority of damage in all sections was a clipped terminal. In block A, 90% of the damage in the control and weasel-scent-treated areas consisted of clipped terminals, and in the pinosylvin-treated area 100% of the damage was clipped terminals. In block B, clipped terminals made up 56% of the damage in the control area, 62% in the pinosylvin-treated area, and 83% in the weasel-scent-treated area. Damage suffered in block B also included a percentage of stems gnawed in each section, which was not seen in block A.

DISCUSSION

This replicated trial has provided statistical evidence that pinosylvin is effective in reducing overwinter feeding damage by meadow voles and snowshoe hares, at least when applied to spring-planted, plug stock, pine seedlings. The results for the weasel scent were less convincing. Although weasel scent produced a significant reduction in damage in block A, in block B the reduction was not statistically different from the control.

The large difference in the amounts of damage in each section when compared between blocks indicates that site-specific factors may play a role in the effectiveness of the deterrents. We postulate that the weasel scent works by causing voles and hares to reduce their feeding in the area due to fear of possible predation; however, if there is a shortage of alternative foods, the voles and hares may risk predation rather than starve. We also believe that pinosylvin works best when voles have an alternative source of nutrition, and, as a result, will avoid the seedlings that smell as though they have a high concentration of antifeedant. If, however, an alternative food is in short supply, voles and hares have very little choice, and, as a result,will feed upon the seedlings. The results of this study indicate that voles and hares may be more likely to risk predation than feed on seedlings with high concentrations of antifeedant.

CONCLUSIONS

During periods of high hare or vole numbers and expeted damage to seedlings, pinosylvin, and, to a lesser extent, weasel semiochemicals, may be a useful method of biological plantation protection. Our results suggest that pinosylvin was highly successful in reducing herbivore feeding damage in the spring-planted sites, and weasel scent, although less effective, was also able to reduce

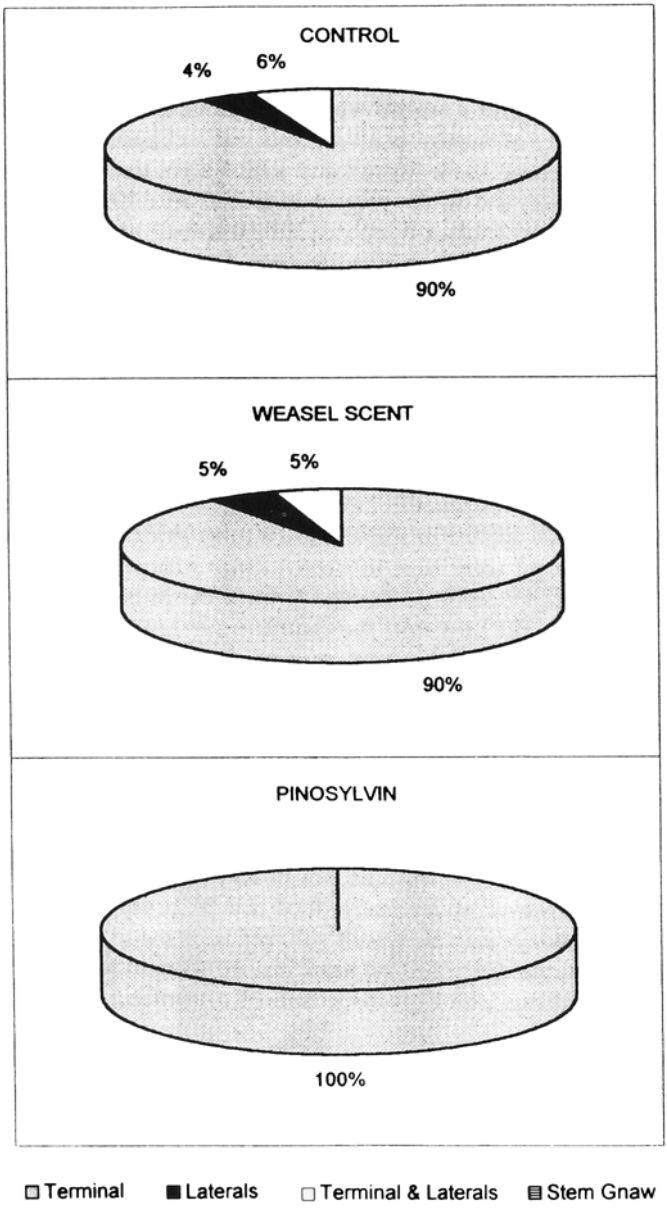

FIG 2. Type of damage in block A.

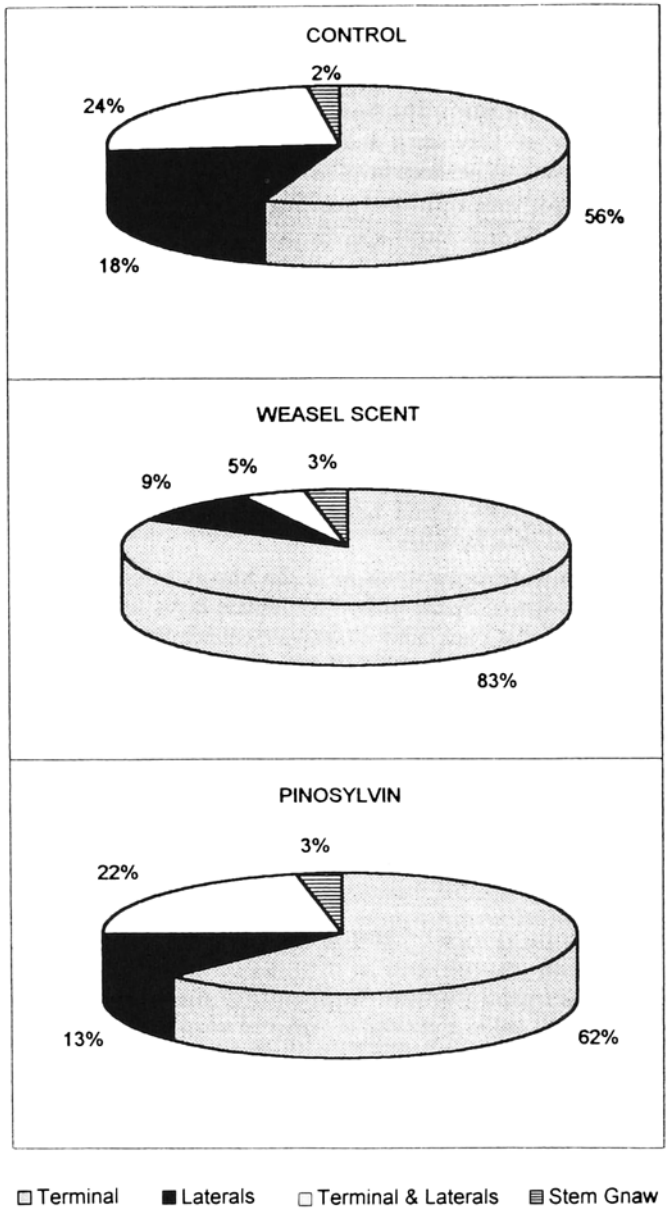

FIG. 3. Type of damage in block B.

feeding damage. These results enhance the desire to use nontoxic methods to control target species and the damage they cause.

Both pinosylvin and synthetic weasel odors have a number of benefits that other deterrents do not have. The first, and in some locations most important, is that the devices are very small and, as a result, have a low visual impact. Secondly, there is no maintenance involved. If attached correctly (devices should be hung on an upper lateral), treated seedlings would not have to be dealt with again. Thirdly, the devices target specific pest species and are not a danger to other wildlife.

The possibility that these herbivores are more likely to risk predation and eat a palatable food source, rather than feed on a source that has a high concentration of antifeedant, raises a number of questions about the role nutrients within the seedlings play in the occurrence of overwinter herbivore feeding damage. If the nutrients responsible for the increased palatability could be identified, then changes in nursery growing regimes might effectively reduce herbivore feeding damage. Pen feeding trials using voles and seedlings grown under different nutrient regimes would be extremely useful in determining which nutrients are keyed onto by voles.

The results also raise questions as to the importance of plant antifeedants in driving the population cycles of both snowshoe hares and meadow voles. It is well documented that plant antifeedants can change significantly after severe browsing (Bryant, 1981; Bryant and Kuropat, 1980; Bryant et al., 1983). Our results indicate that high concentrations of antifeedant may be capable of causing shifts in the feeding behavior of voles and hares to areas of higher predation risk.

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TEMPORAL CLUMPING OF BARK BEETLE ARRIVAL AT PHEROMONE TRAPS: MODELING ANEMOTAXIS IN CHAOTIC PLUMES

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Abstract—The sequence of arrival of the bark beetles *Ips typographus* and *Pityogenes chalcographus* (Coleoptera: Scolytidae) at traps baited with their synthetic pheromones was monitored with a portable fraction collector. Histograms of the natural arrival patterns of both species were nonrandom and clumped at shorter time scales (1-, 2-, 4-, 5-, or 6-min cells) but appeared random at larger time scales (10, 20 or 30 min). Monte Carlo generation of similar histograms showed them to be random at all of these time scales. A stochastic computer model could graphically simulate insect orientation to odor sources based on present theories of odor-modulated anemotaxis and casting. Although this model was used throughout, since it assumes only that insects cast perpendicular to the current wind direction, a second model could slightly improve orientation success. However, the second model requires that the insect remember its ground path (upwind) prior to losing the plume (after an abrupt wind direction change). The effects of casting and flight parameters on orientation success and randomness of arrival sequence within various plumes were determined by simulation. Similarly, the effects of random walks in plume direction, plume width, and wind speed were explored. The results showed that dynamic random variations in plume direction and especially wind speed could cause an otherwise random arrival sequence (e.g., under constant wind) to become clumped and nonrandom. Therefore, the clumped arrival patterns of bark beetles and other insects, including *Spodoptera litura*, at pheromone sources could result from random-walk fluctuations in wind speed and wind direction.

Key Words—Orientation, attraction, odor-modulated anemotaxis, pheromone plumes, casting, simulation models, Coleoptera, Scolytidae, Lepidoptera, Anemotaxis.

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INTRODUCTION

In 1984, spruce bark beetles, *Ips typographus* (Coleoptera: Scolytidae), were collected by a fraction collector as they arrived at a trap baited with synthetic pheromone (Byers and Löfqvist, 1989). It appeared that the arrival of these beetles at the trap occurred in groups and not uniformly random during an hour with relatively constant temperature. I questioned this, however, because Ripley (1981) stated that a random pattern, such as a "Poisson forest" or the stars in the sky, appears to a casual observer as a nonrandom pattern of groups (e.g., the mythological constellations). On the other hand, it is reasonable to suppose that nonrandom patterns of arrival could result from difficulties of insects to orient in pheromone plumes under certain meteorological conditions. Thus, the first objective here was to determine whether individuals of two species of bark beetle arrive at pheromone-baited traps in a nonrandom, clumped sequence.

The optomotor anemotaxis mechanism for orientating to phermone sources proposed for insects, especially moths (Kennedy, 1939, 1983; David et al., 1982; Baker and Haynes, 1987; Baker, 1989), probably also functions in bark beetles (Choudhury and Kennedy, 1980). In this theory, an insect attempts to fly directly upwind when in contact with a packet of pheromone-laden air of the plume, but casts (flying back and forth perpendicular to the wind) when contact is lost. In contrast to walking insects, insects that are flying probably cannot use mechanoreceptors to sense the wind direction. However, flying insects can perceive wind direction by observing the ground below: in head-on wind, the ground moves directly underneath during flight. If the visual ground field moves from right to left somewhat, for example, then wind is coming from the left. The insect thus turns to the left to minimize the transverse ground shift and keep the ground moving directly underneath in order to head upwind toward the pheromone source. However, it is not known if all insect species use the same orientation mechanisms when flying within or on the edge of a pheromone plume.

The seminal paper of David et al. (1982) advanced our thinking about how flying insects reach a pheromone source in the field. Their theory, called "odor-modulated anemotaxis," asserts that optomotor anemotaxis and casting are sufficient to cause orientation to the odor source. They showed that wind direction may change rapidly causing a "snaking" of the plume, much as one might spray water from a hose. This occurs because the volume of air passing the odor source continues in a straight line downwind—and thus any insects encountering this volume will experience a wind direction flow that is directly opposite the direction to the source. They maintain that all the insect has to do is fly upwind, using optomotor anemotaxis when detecting pheromone, and cast when not detecting pheromone in the expectation of reentering the plume.

Central to our understanding of orientation is the structure of the pheromone

plume—in which our knowledge also has gone through an evolution. Plumes were visualized earlier as time-averaged Gaussian plumes extending up to several kilometers (Bossert and Wilson, 1963). Other Gaussian plume models have been proposed (Fares et al., 1980), but these and others did not reliably predict when gypsy moth wing-fanning would occur in relation to the mean wind direction (Elkinton et al., 1984). Since then the idea of a filamentous and snaking plume has become the paradigm (Murlis and Jones, 1981; David et al., 1982; Elkinton and Cardé, 1984). The idea of the "active zone" (Bossert and Wilson, 1963) or "active space" (Nakamura and Kawasaki, 1977, 1984; Baker and Roelofs, 1981) is also central to our concept of a plume. Within the active space, the concentration of pheromone molecules is sufficient to elicit a behavioral response in an insect, and this is the area that we spatially visualize as the plume.

My second objective was to make a computer model to simulate the odor-modulated anemotaxis and casting mechanisms associated with a plume in order to understand better how flying insects find a pheromone source. Using Monte Carlo randomization methods, several plume- and insect-related parameters were simulated over wide ranges (overlapping most natural conditions) to help understand their individual effects on insect attraction to pheromone. The third objective was to discover whether certain meterological parameters that varied at random in the simulation model could frequently produce nonrandom, clumped arrival patterns similar to those observed in nature.

METHODS AND MATERIALS

Successive Catches of Bark Beetles Attracted to Pheromone Components. A portable fraction collector similar to that used earlier (Byers, 1983), but with a crystal-controlled timer (Byers, unpublished), was used to sequentially collect flying bark beetles in 1- or 2-min periods during their attraction to pheromone baits. *Ips typographus* were collected this way during one to several hours in a Norway spruce forest clear-cut located in Grib Skov, 30 km northwest of Copenhagen, Denmark (May 29 and June 5, 1984), as they oriented to drain-pipe traps (with funnel) releasing 2-methyl-3-buten-2-ol (50 mg/day) and (1*S*, 4*S*, 5*S*)-*cis*-verbenol (1 mg/day) (Bakke et al., 1977, 1983; Byers et al., 1988). The fraction collector with only a 30-cm-diameter funnel was used to simultaneously collect *I. typographus* and *Pityogenes chalcographus* attracted by pheromone components of both species in another Norway spruce forest clear-cut in Grib Skov (June 16–17, 1987). *P. chalcographus* was attracted by baits releasing chalcogran (46:54 *E*:*Z*, 98% pure from W. Francke, University of Hamburg, Germany) released at 1 mg/day and methyl (*E*, *Z*)-2, 4-decadienoate (99.5% pure, Cyanamid Agrar) at 0.1 mg/day (Francke et al., 1977; Byers et al., 1988,

1990). During collections, the pipe trap + funnel or funnel was strongly tapped every 10–30 sec to ensure that all beetles were immediately collected after landing. Sex and numbers of beetles collected in the periods were determined in the laboratory.

Simulation of Insect Movement during Orientation and Casting in Pheromone Plumes under Windy Conditions. The simulation model (Figure 1) requires 30 input parameters (in units of meters, seconds, degrees, or as indicated). Twenty of these parameters can be varied systematically over a number of simulations. The parameters and their usual values, unless varied during simulation, were: (1) number of simulations per step variable, 8 or 16; (2) number of insects, 50; (3) *x*-axis, 216; (4) *y*-axis, 162; (5) plume length, 100; (6) flight speed in plume, 2; (7) cast proportion, 0.5; (8) number of castings, 2; (9) checks per cast, 20; (10) flight speed outside plume, 2; (11) trap radius, 0.25; (12) minimum wind speed, 0 or 1; (13) maximum wind speed, 1 or 2.5; (14) minimum wind speed enabling anemotaxis, 0.2; (15) maximum wind speed changes/min, 0, 1, 10 or 30; (16) maximum wind speed change per change, 0 or 0.5; (17) angle of maximum turn in plume, 20; (18) angle of maximum turn outside plume, 20; (19) probability of orienting during move when in plume, 1: (20) maximum plume angle, 90 or 160; (21) minimum plume angle, 20 or 90; (22) maximum plume angle changes/min, 0 or 10; (23) maximum plume angle change per change, 0 or 5; (24) maximum plume width angle, 10; (25) minimum plume width angle, 10; (26) maximum plume width angle changes/min, 0 or 5; (27) maximum plume width angle change per change, 0 or 5; (28) number moves per time period, 300; (29) total time periods, 12; and (30) random seed number, varied.

The complexity of the model can be reduced by choosing parameters that are realistic, such as *x* and *y*-axes, plume length, insect number per area, flight speeds, and turning angles (usually ≤ 20 degrees). Variations in other parameters often have little effect or may be optimized, such as cast proportion, number of casts, and number of checks per cast. Many parameters can be ignored by making them equal zero. Other effects can be held constant by setting two parameters equal, for example, minimum and maximum wind speeds both set to 1 m/sec.

The general algorithms for simulation of insect movement and catch by traps have been described in Byers (1991, 1993). A plume in the models is defined geometrically as a pie slice of a circle with width equal to the arc in degrees and length equal to the radius (Sabelis and Schippers, 1984). Insects not in contact with the plume take steps in a forward direction with possible random deviations up to an angle of maximum turn (either right or left at random). Moves are based on either of two speeds (or distances covered in 1 sec) when either inside or outside the plume. However, insects can have less ground speed when casting or when countered by the wind. The ground path

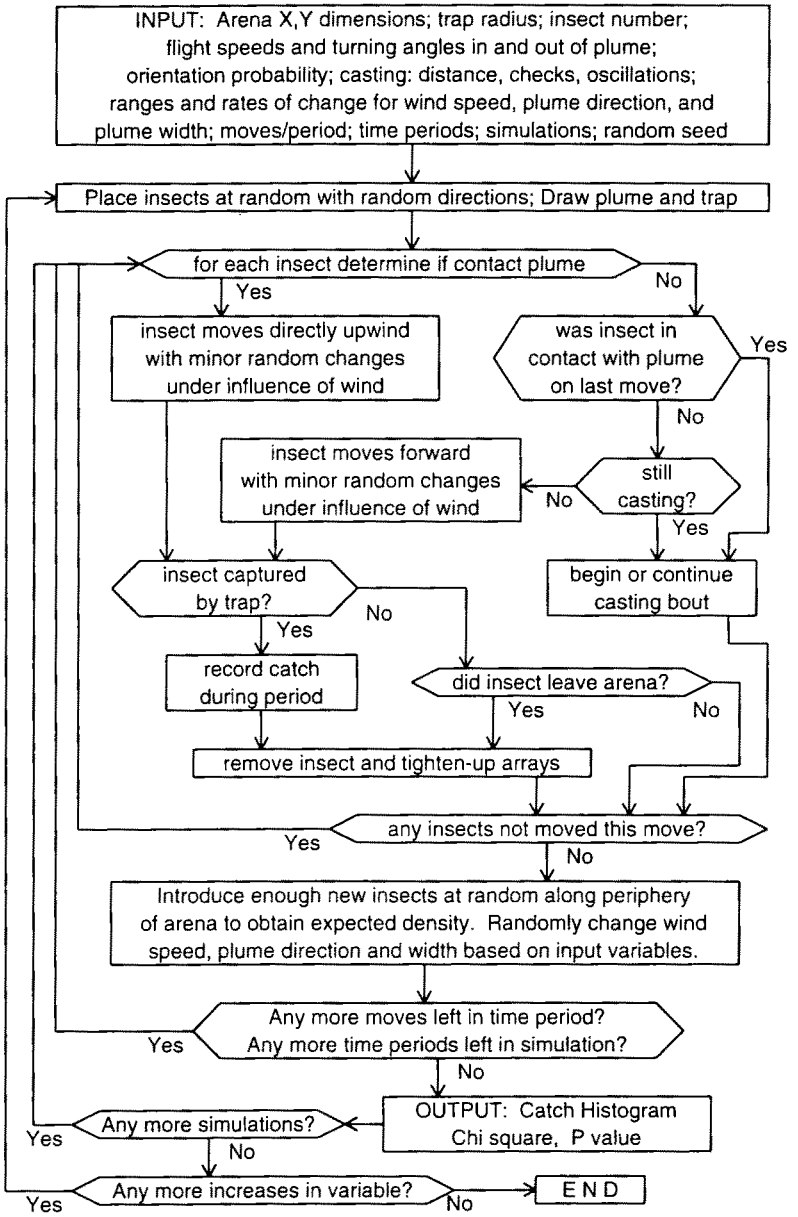


FIG. 1. Flow diagram of graphical computer model for orientation of insects toward pheromone-baited traps while flying in changing wind and plume structure.

of an insect is determined by first calculating the insect vector using polar coordinates of insect flight speed and former direction (or upwind direction if in the plume) plus the random angle of turn, and then adding the wind vector, based on the polar coordinates of wind direction and speed. Insects that land in or cross the trap during a move are caught (Byers, 1991) and recorded for the time period. During a move, any insects that are caught or leave the arena are removed (Figure 1). After all insects have moved a step, the number of insects remaining outside the plume that are not casting is counted. If this number is less than the expected number based on the initial density (minus the plume area), then enough insects are added at the arena's periphery (in proportion to the dimensions) to attain the desired density (Figure 1). This ensures that a relatively constant density of insects surrounds the plume under any conditions.

Modeled insects cast after they step out of a plume, through a plume, or when the plume moves away between insect moves (Figure 1). The algorithm for determining whether an insect is within the plume is based on whether the insect's angle to the source is within the plume angles and that the insect is closer to the source than the plume length. Near the source as the plume narrows, it is possible for insects to "step over the plume," so a second algorithm is used to determine if the insect's move segment intersects either plume segment (Byers, 1992). When an insect either steps over the plume or leaves the plume, it does not begin casting until the next move, whereupon it turns perpendicular to the wind direction by turning either right or left at random.

The distance of a cast, defined as the maximum length of a perpendicular excursion, can be specified as any proportion P of the distance covered in 1 sec while in the plume (flight speed within the plume). The insect takes one move to travel the cast distance and one move to return to the starting point. The insect checks to see whether it is in the plume at several points spaced equidistantly along the cast path. The number of checks can be varied but was usually held constant at 20 in the simulations here. If the insect encounters the plume while casting at any of the check points, it stops at this position and on the next move begins to fly upwind again (Figure 1).

Assuming the plume is not found during a cast and return, the casting oscillation continues until the insect takes a cast in the direction opposite the initial one (a third move) and then returns to the start on the fourth move. More than one casting oscillation can be specified during a bout of casting (in the simulations the default was two oscillations). After casting is completed without success, the insect wanders away to possibly blunder into the plume nearby. This casting algorithm causes insects to orient reliably to the pheromone source except when the plume is very narrow (a few degrees) or very wide. The maximum width of the plume (in degrees) that should allow reliable plume following is calculated from the equation:

$$2\left\{\frac{\pi}{2} - \arcsin\left[\frac{A \sin\left(\frac{\pi}{2} - \alpha\right)}{\sqrt{A^2 + B^2 - 2AB\cos\left(\frac{\pi}{2} - \alpha\right)}}\right]\right\}\frac{180}{\pi} \quad (1)$$

with $A = S - [W/\cos(\alpha)]$ and $B = P\,S - W\tan(\alpha)$ where S is the flight speed (meters per second) within the plume, W is the wind speed (meters per second), P is the cast proportion (≤ 1), and α is the angle of maximum turn within the plume (in radians). The largest α possible for casting to function reliably is limited by the cast proportion according to:

$$\alpha_{max} < \arcsin(P)\frac{180}{\pi} \quad \text{where} \quad \arcsin(P) = ATN\left(\frac{P}{\sqrt{1 - P^2}}\right) \quad (2)$$

The α used in simulations should be less than α_{max} since this angle assumes the most narrow plume possible. The *arcsin* transformation (equation 2), uses the *ATN* (arctan) function of BASIC and should also be used with equation 1.

To understand the effects of certain parameters on catch in simulations, several combinations of parameters were held constant while the magnitude of one parameter was increased stepwise, with 8 or 16 replicates per step and each replicate having 12 time periods consisting of 5 min (300 moves). Attempts were made to simulate the frequency distributions of natural catches by allowing several meteorological parameters to vary at random during simulations. These were wind speed, plume direction angle (wind direction), and plume width. All three variables have two components that can also be varied at random, namely, probable number of changes per minute and maximum random change at each change. The probable number of specified changes, e.g., 10 changes in wind speed per minute, is determined at each move (equal to 1 sec) by whether the random number generated by computer (an eight-digit number < 1) is less than 10/60.

The arrival patterns of bark beetles were analyzed for randomness over different time periods by making histograms of the catches using various cell widths (1–30 min). These histograms were compared to average expected catches based on the total catch divided by the number of histogram cells using chi-square analysis. To check the method, histograms of the same number of cells and total catch were generated by Monte Carlo methods and compared similarly. The catches in the computer simulations were also grouped in 5-min periods and analyzed by chi-square, whereupon exact P values were calculated and averaged for graphical and statistical analyses. Points with bars in the figures

represent upper and lower SEM from 8 to 16 simulation runs (however, some graphs have no bars to reduce complexity). Curves were fitted to the data with the following priority of methods: linear and nonlinear regression, combinations of these, equation fitting by eye, moving average, and connection of points.

RESULTS

Successive Catches of Bark Beetles Attracted to Pheromone Components. A total of 108 (27.8% males) *I. typographus* were collected in 2-min periods over 3.5 hr beginning at 14:30 hr, on May 29, 1984. The pattern of arrival, as shown in a histogram of 5-min periods (Figure 2) summarizing the actual counts (dots), was significantly different from an expected distribution based on a uniform random arrival ($P = 0.028$, chi-square). A Monte Carlo-generated histogram of the same total count shown for comparison was not different, as expected from a uniform random arrival ($P = 0.664$) nor was an average of 10 such distributions (Figure 2). When the natural data and the randomly generated patterns were subdivided into cells of different durations (1–30 min) and ana-

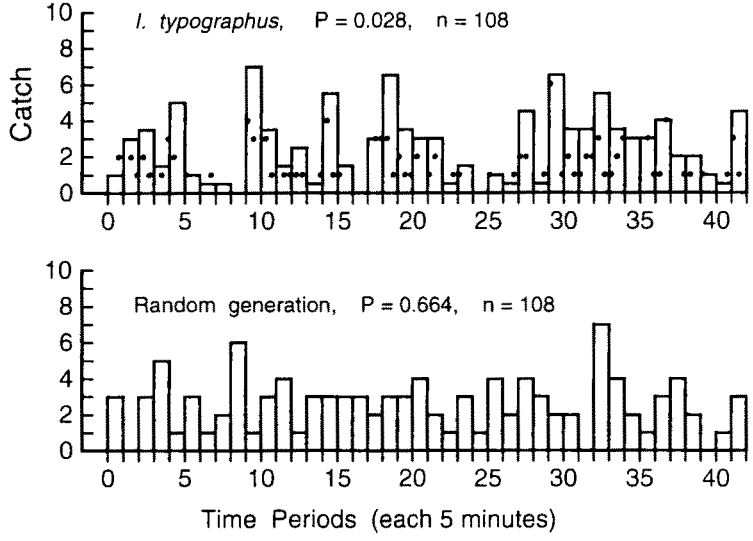


FIG. 2. Top: histogram of *Ips typographus* arrival at pheromone-baited traps (5-min periods over 3.5 hr beginning 14:30 on May 29, 1984; actual 2-min catches represented by dots). Bottom: histogram of Monte Carlo-generated random distributions of the same number of periods and total catch (108). Average P value for 10 generated distributions was 0.56 ± 0.28 (±95% CL).

lyzed by chi-square, only the natural data were significantly nonrandom at 2-, 4-, and 5-min periods, while larger lumpings of the data in cells of 10 or more minutes indicated a relatively constant catch during the whole period (Table 1). On June 5, 1984, 62 (16.1% males) *I. typographus* were caught over an hour period (Table 1) and histograms of cells of 2–6 min also were significantly different from random while larger groupings were not (Table 1).

Over a 40-min period of 2-min collections, 226 (40.7% males) *P. chalcographus* were caught, and the histogram pattern (Figure 3) was significantly different from an expected distribution of random arrival ($P < 0.001$). Similar Monte Carlo-generated distributions, however, were not different from random as analyzed by chi-square (Figure 3). In two other 40-min periods of 1-min collections, 93 (44.1% males) and 81 (39.5%) *P. chalcographus* were caught, and the histograms were significantly different from expected random arrivals at several different lumpings of the data from 1 to 10 min (Table 1). As expected, similar chi-square analyses of Monte Carlo-generated histograms rarely ($\leq 5\%$ cases) were found to be different from random.

The temperature during the 40-min collection periods varied less than 2° and was generally optimal for flight (21–25°C). Winds, measured occasionally with a fan anemometer, varied between 0.8 and 2.3 m/sec (average for 20 sec). Wind direction was observed with a wind vane to change direction up to 60° within a second or two while generally prevailing in one direction (although direction reversals were observed). It appeared that most beetles arrived during lulls in the wind.

Simulation of Insect Movement during Orientation and Casting in Pheromone Plumes under Windy Conditions. The computer model shows on screen whether the parameters specified allow simulated insect orientation toward a pheromone source. When the model was tested under constant conditions (parameters as in Figure 4), the effect of enlarging the width of the plume from 0 to 9° caused a cumulative exponential increase in catch (Figure 4). Thereafter, an increase in plume width up to about 40° caused a linear increase in catch. In theory, the catch should be linearly proportional to the perimeter length of the plume, i.e., a 90° plume will catch twice as many as a 45° plume of the same radius since the arcs are proportional to the plume angles. However, as seen from equation 1, a plume width of more than 39.53° does not allow reliable casting with the parameters specified in Figure 4, and thus the catch declined exponentially ($Y = ae^{-bX}$) above this angular width (Figure 4). The arrival patterns in the simulations at all plume widths had average P values well above significant levels, indicating that arrival was random as should be expected (Figure 4).

Several parameters involving orientation such as checks per cast, casting oscillations, angle of maximum turn within the plume, and probability of orientation at each step were investigated for their affects on arrival at traps. During

TABLE 1. CHI-SQUARE ANALYSIS OF BARK BEETLE ARRIVALS AT PHEROMONE-BAITED TRAPS IN THE FIELD[a]

	Minutes per histogram cell							
	1	2	4	5	6	10	20	30
Ips typographus								
May 29, 1984 (14:30)								
df		104	51	41	34	20	9	6
P		0.005	0.004	0.028	0.054	0.326	0.518	0.607
June 5, 1984 (14:30)								
df		29	14	11	9	5	2	1
P		0.004	0.002	0.063	0.007	0.310	0.290	0.450
Pityogenes chalcographus								
June 16, 1987 (14:30)								
df		19	9	7	5	3	1	
P		<0.001	<0.001	0.016	0.013	0.141	0.033	
June 16, 1987 (15:18)								
df	19	9	4	3	2	1		
P	<0.001	<0.001	0.002	<0.001	0.002	0.003		
June 17, 1987 (14:46)								
df	19	9	4	3	2	1		
P	0.009	0.134	0.015	0.018	0.068	0.222		

[a]The arrival patterns were partitioned into various histograms of different cell widths (1–30 min) and compared to average expected frequencies based on total catches using chi-square analysis. $P < 0.05$ indicates that a distribution of catch is not uniformly random.

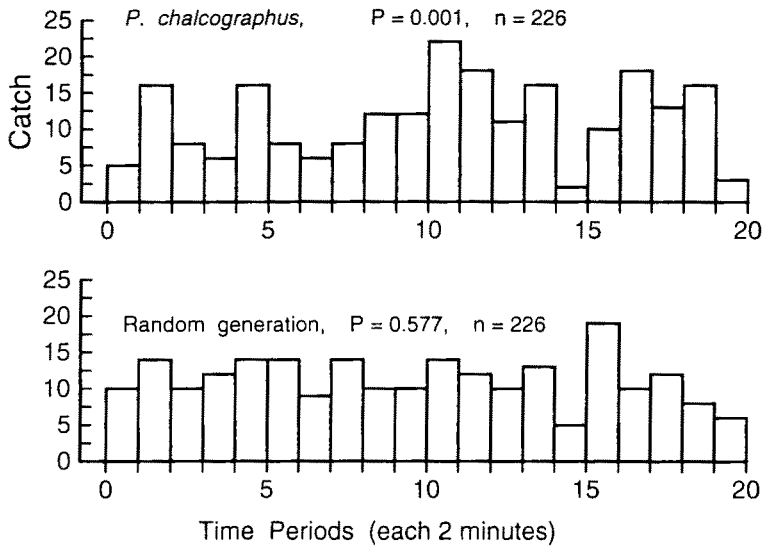


FIG. 3. Top: histogram of *Pityogenes chalcographus* arrival at pheromone-baited traps (2-min periods over 40 min beginning 14:30 on June 16, 1987). Bottom: histogram of Monte Carlo-generated random distributions of the same number of periods and total catch (226). Average *P* value for 10 generated distributions was 0.58 ± 0.16 (±95% CL).

a cast an insect must sample the air for pheromone; this can be done only once at the outermost extent of a cast, or many times (equally spaced) along the path of a cast. The orientation success or catch increased with checks per cast approximately in an exponentially hyperbolic relationship [$Y = ae^{(b/X)}$] while having no apparent affect on the randomness of arrival (Figure 5A). This relationship was apparent even with 0.5° plume widths, 0.025 m trap radius, and finer 0.1-sec insect moves. An increase in the number of casting oscillations had no affect on orientation when plume direction remained constant. When the plume was allowed to shift direction at random (10 times per minute), the orientation success became poorer as the random swing became larger (maximum 1, 5, or 10°). However, orientation success increased as the number of casting oscillations increased, the more so the larger the random swing (Figure 5B). No effects on the randomness of arrival were found due to the number of casting oscillations.

An increase in the angle of maximum turn from 0 to 27.8° has little affect on orientation success (Figure 5C), and this is expected based on equation 1 for a 10° wide plume. A plume width above 30° does not allow reliable orientation as seen from equation 2. Across the spectrum of possible insect turn angles,

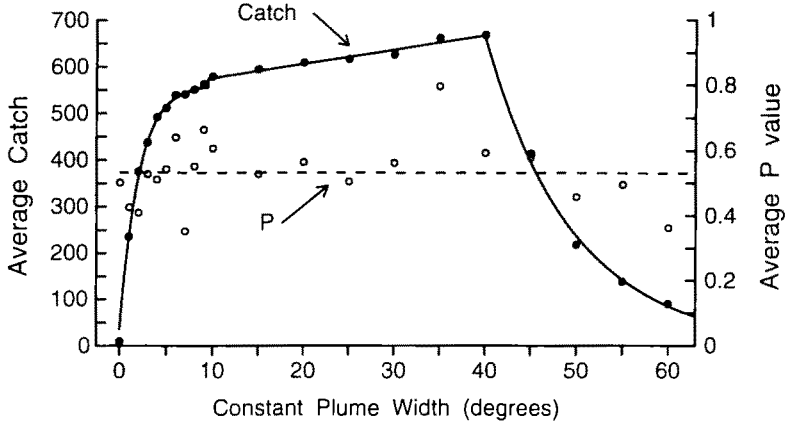
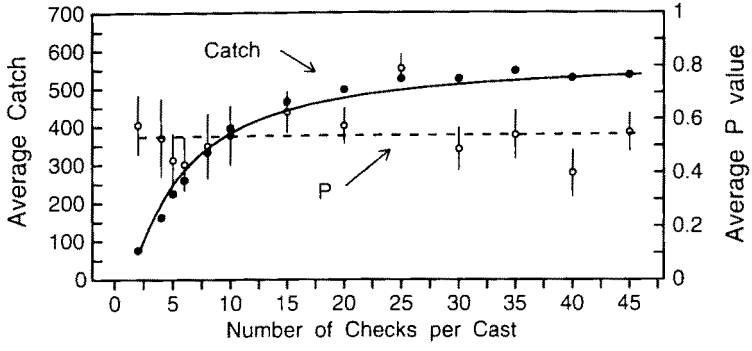
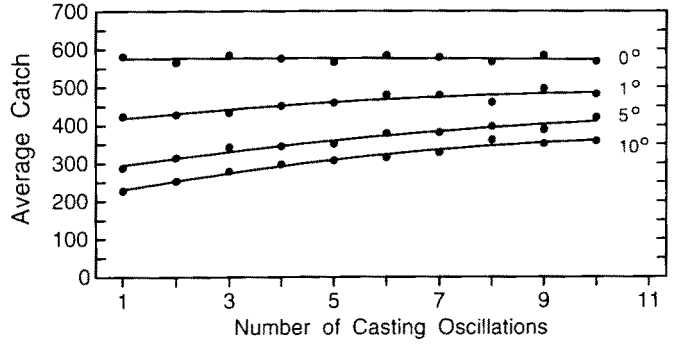


FIG. 4. Effect of plume width angle on the average catch and the average *P* value (chi-square analysis of arrival histogram) over an hour of simulated time. Simulations were performed 16 times at each plume width under the following conditions: 216 × 162-m arena; 100-m plume length; 50 insects; 2 m/sec flight speed both inside and outside plume; 0.25 m trap radius; 1 m/sec constant wind; 20° angle of maximum turn both inside and outside plume; probability of 1 to orient at each step within the plume; 0.5 casting proportion of flight speed within plume; 20 checks per cast; 2 casting oscillations; 90° constant plume direction; plume width angle was varied; constant plume width during a simulation; 300 moves/time period (5 min); and 12 time periods (total 1 hr) per simulation.

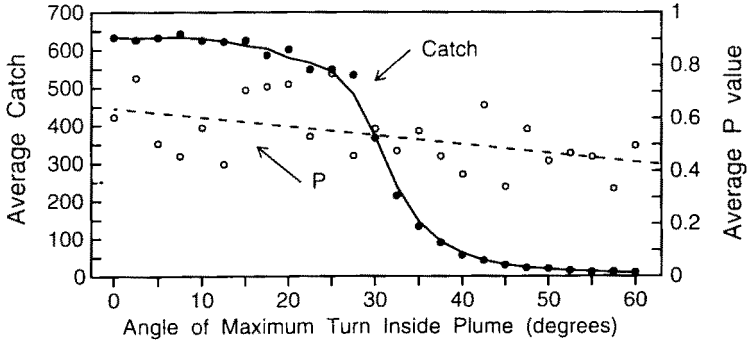
FIG. 5. (A) Effect of number of casting checks per cast on the average catch and the average *P* value (chi-square analysis of arrival histogram) over an hour of simulated time. Simulations were performed eight times at each value under the model parameters in Figure 4, except as indicated, and plume width angle was held constant at 5°. (B) Effect of number of casting oscillations on the average catch and the average *P* value (chi-square analysis of arrival histogram) over an hour of simulated time when the plume direction was allowed to vary in direction at 10 directional changes per minute (at random) at a maximum change of 0, 1, 5 or 10° at random. Simulations were performed eight times at each value under the model parameters in Figure 4, except as indicated, and plume direction could range from 20 to 160° while plume width was constant at 10°. (C) Effect of the insect's angle of maximum turn within the plume on the average catch and the average *P* value (chi-square analysis of arrival histogram) over an hour of simulated time. Simulations were performed eight times at each value under the model parameters in Figure 4, except as indicated, and plume width was constant at 10°.


Average Catch
Average P value
Catch
P
Number of Checks per Cast

A.


Average Catch
0°
1°
5°
10°
Number of Casting Oscillations

B.


Average Catch
Average P value
Catch
P
Angle of Maximum Turn Inside Plume (degrees)

C.

there was little effect on patterns of arrival since all average P values indicated no differences from random (Figure 5C). The probability of orientation at each step was usually held constant at 1; however, lower probabilities increase the stochasticity and look more realistic on screen. Although no figure is shown, the average catch (Y) increased form 0 to 600 in relation to the square of the probability of orientation at each step ($0 \leq P_0 \leq 1$) according to $Y = 9.36 + 563.4P_0^2 (r^2 = 1$, parameters as in Figure 4). Again, average P values indicated random arrivals at all orientation probabilities.

The rate of catch decreased exponentially (best fit was a Taylor exponential: $Y = e^{a-\sqrt{X}}$) with an increase in the maximum random change in plume direction random walk, when the direction was changed at either 1, 10, or 30 times per minute at random (Figure 6). The catch rate also decreased similarly with an increase in the rate of changes in plume direction (at a maximum random change of 2° per change, figure not shown). Surprisingly, random changes in plume direction (10/min or more) produced a random arrival of insects at the trap over time, except when only a few changes per minute occurred (e.g., 1/min, Figure 6).

The rate of catch declined as a Taylor exponential ($Y = e^{a+b\sqrt{X}}$) with an increase in plume width changes per minute (each change a maximum of 2° at random) when the plume width could vary between 10 and 20° or 10 and 60° (Figure 7). The arrival of insects was different from random when plume widths could vary between 10 and 60° and the number of width changes were greater than about 10/min, but no effects were observed when plume width was restricted in a more realistic range between 10 and 20° (Figure 7).

In preliminary models, attempts to maintain a constant number of insects in the arena were done by replacing any that left the arena or were caught. However, under wind speeds approaching the flight speed, insects accumulated in the plume as they moved slowly toward the source, thus depleting the density surrounding the plume. Since there were less insects available to contact the plume, catch rate decreased with time. In reality, in an ocean of constant insect density, the same density surrounding the plume should persist at any wind speed. Thus, the model was modified so that any insects that either entered the plume (or were casting), left the arena, or were caught were replaced on the arena's perimeter to obtain the specified density. In this case, at higher wind speeds, insects move more slowly toward the source, but the accumulation of insects within the plume perfectly compensates so the rate of catch remains constant, until just below the flight speed when orientation is impossible. The wind speed W at which the average insect within the plume cannot move upwind is determined by the following equation:

$$W = \int_0^{\alpha} S \cos(\beta) \approx \sum S \cos(\beta) \quad \text{and} \quad \beta = 0, 0.0001, \cdots, \alpha \qquad (3)$$

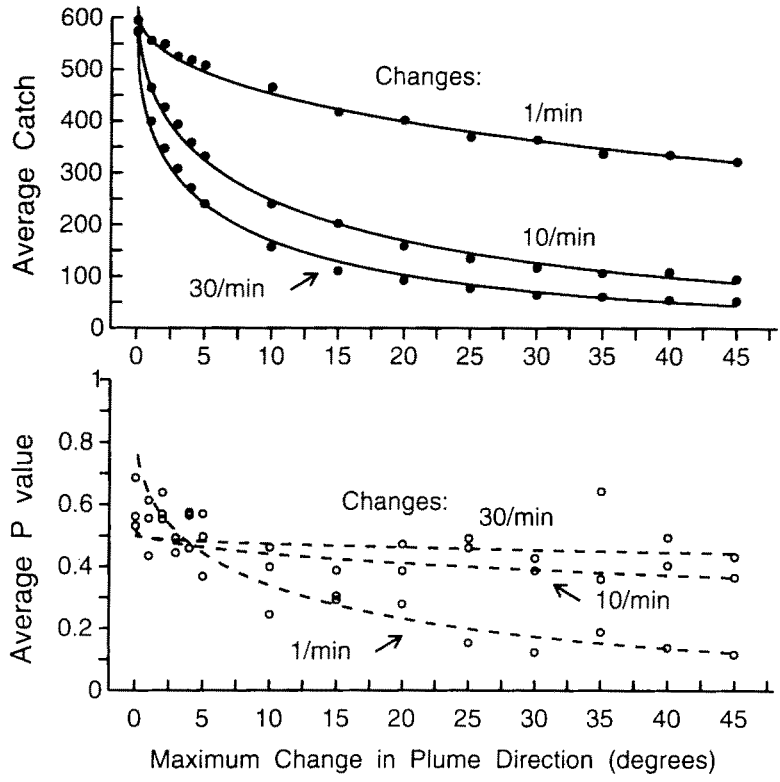


FIG. 6. Effect of maximum change in a random walk in plume direction (1, 10, and 30 changes per minute at random) on the average catch (top graph) and the average P value (bottom graph) (chi-square analysis of arrival histogram) over an hour of simulated time. Simulations were performed 16 times at each value under the model parameters in Figure 4, except as indicated, and plume direction could range from 20 to 160° while plume width was constant at 10°.

where S is the speed within the plume (e.g., 2m/sec) and α is the maximum angle of turn (e.g., 20° in radians).

Variable winds (random walks) over a range of 0 to 2.5 m/sec had little or no effect on rate of catch but did cause the pattern of arrival to become nonrandom or clumped. The arrival pattern swiftly became nonrandom with small increases from 0 to 0.2 m/sec in the maximum wind speed change (at random) occurring approximately 30 times per minute (at random) but then gradually became less clumped and more random with further increases above 0.5 m/sec in maximum changes (Figure 8). The minimum wind speed that

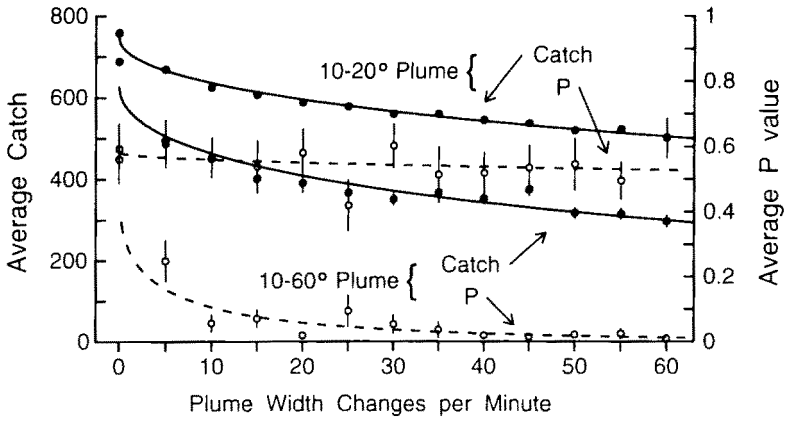


FIG. 7. Effect of number of plume width changes per minute (taken at random) in a random walk at a maximum of 2° per change (at random) on the average catch and the average P value (chi-square analysis of arrival histogram) over an hour of simulated time. Two simulation series were done, one in which the plume width could vary from 10 to 20° and the other with an allowed range from 10 to 60°. Simulations were performed 16 times at each value under the model parameters in Figure 4, except as indicated, and the angle of maximum turn, normally 20°, was reduced to 10° to allow reliable casting on wide plumes.

enabled anemotaxis was set at 0.2 m/sec, assuming upwind orientation is not possible without some air movement (Nakamura and Kawasaki, 1984). Below this wind speed insects in contact with pheromone odor continued to move forward with random deviations like those outside the plume.

Wind speed fluctuations that occurred about 30 times per minute at changes of up to 0.5 m/sec caused the pattern of arrival (Figure 9) to be nonrandom ($P < 0.002$). It appears that catch rate is higher during a lull period following a time with higher winds (Figure 9), although the 5-min rates depend on a complex interaction between wind speed, time, and direction of the speed change.

DISCUSSION

The models here may help us to define, organize, and understand orientation concepts as well as communicate and test that understanding in order to make predictions and comparisons (Worner, 1991). The model has fewer conceptual problems when wind direction, speed, and plume dimensions are held constant at reasonable levels. Objections arise when plume width or direction vary. For example, modeled plumes change direction along their entire length while wind

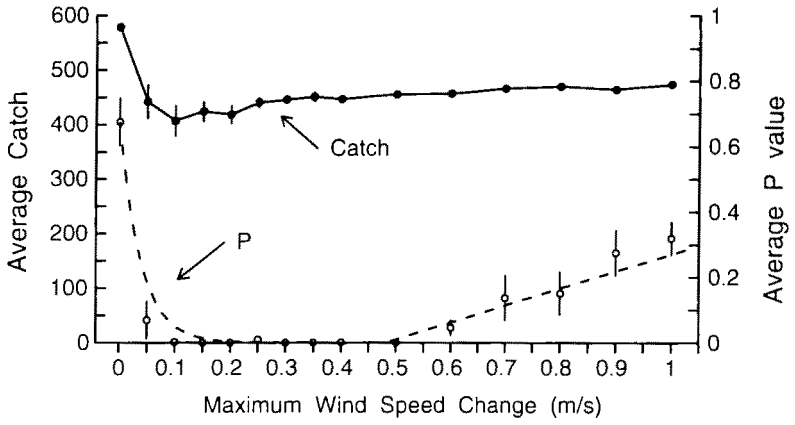


FIG. 8. Effect of maximum change at random in a random walk in wind speed (about 30 changes per minute at random) on the average catch and the average P value (chi-square analysis of arrival histogram) over an hour of simulated time. Simulations were performed 16 times at each value under the model parameters in Figure 4, except as indicated, and wind speed could range from 0 to 2.5 m/sec while plume width was constant at 10°.

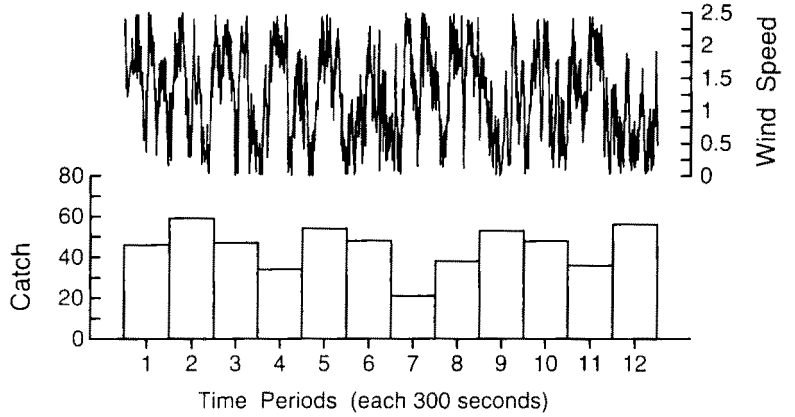


FIG. 9. Top: Wind speed fluctuations during an hour of simulated time in which wind speed could change on average 30 times per minute at a maximum change of 0.5 m/sec at random within a random-walk range between 0 and 2.5 m/sec. The simulation was performed under the model parameters in Figure 8. Bottom: The corresponding histogram of simulated catch, the arrival sequence was significantly different from an expected frequency by chi-square analysis, $P < .002$.

speed and direction changes affect all areas of the arena simultaneously. More realistic snaking plumes are the subject of future models. However, the rate of successful orientations within a snaking plume and the swinging plume here may not be significantly different since an insect would experience odor puffs of the same periodicity and dimension in both models. Granted, only the snaking plume would allow realistic coordinated movements among insects in the arena, but this seems unimportant to arrival rates.

The model density of 50 beetles in a 216 × 162-m arena was 16% of the natural flight density in one spruce clear-cut in Denmark (Byers et al., 1989), but of course natural densities must vary greatly. In any case, the relationships and randomness of arrival patterns found here do not depend on insect density. The trap radius of 0.25 m used in the model is larger than most tree trunks, but probably smaller than the visual attraction radius of many bark beetles to trees under colonization (Tilden et al., 1983; Lindgren et al., 1983). Again, this is not important to the general relationships and temporal arrival patterns, but only in regard to the absolute catches.

In all simulation series, a period of time to reach equilibrium conditions was allowed before beginning to collect arrival data. Further, in a simulation series each simulation served as the equilibrium period for the next simulation, i.e., all subsequent simulations used the same insects and plume dimensions that existed at the end of the preceding simulation (Figure 1). Attaining equilibrium was especially important at wind speeds approaching the flight speed, where many insects, sometimes thousands, could accumulate within the plume.

An increase in the magnitude of plume direction change occurring relatively often (10–30/min) did not have a significant affect on the randomness of arrival (Figure 6), even though the catch decreased with an increase in the magnitude of directional changes. Many changes in plume direction per minute, each of small magnitude, seems realistic, compared to larger directional changes, since the entire plume jumps in the model. Conceptually, a large shift in wind direction might be acceptable if it occurred rarely, since in this case the plume position is usually stationary. In this case, when the plume took major swings in direction only once per minute on average at random, there were more simulations where arrival was not random, increasingly so with larger directional swings (Figure 6). In nature, changes in wind direction might further affect the arrival patterns since the plume could sweep over brood trees or hibernation sites periodically.

Observations of male *Grapholita molesta* moths flying to a pheromone source in the field under shifting winds convinced Von Keyserlingk (1984) that males losing the plume allowed themselves to drift or be pushed over to the plume's new position by the wind. Baker and Haynes (1987) did not find evidence to support this phenomenon; instead, upon leaving a plume, males seemed to "take up an angle approximately 90° across the windline even as the windline

was in the process of shifting." In the simulations here, insects that lost the plume after a wind shift begin casting across the windline, and if unsuccessful in finding the plume, they took their first step forward against the new wind direction (model 1, Figure 10). In a second model (model 2, Figure 10), insects reached the odor source more often in variable winds because, upon losing the plume, they moved forward initially against the previous wind direction (they would have to remember their immediate ground path).

The reason model 2 insects are more successful in reentering the plume is they have a vector (previous wind direction before wind shift) that adds to the new plume/wind direction vector causing the insects generally to veer toward the new plume position. Whether plume direction changes rapidly enough in nature to afford an adaptive benefit to model 2 insects remains an open question (Figure 10). Which of these two models insects use should be investigated. Namely, do the initial paths taken after casting progress in the former wind direction before wind shifting, or in the new wind direction?

Changes in plume width can be likened to turbulence. Only when the plume was allowed to broaden or narrow over an unrealistically large range (10–60°) could changes in width affect the randomness of arrival (Figure 7). This non-

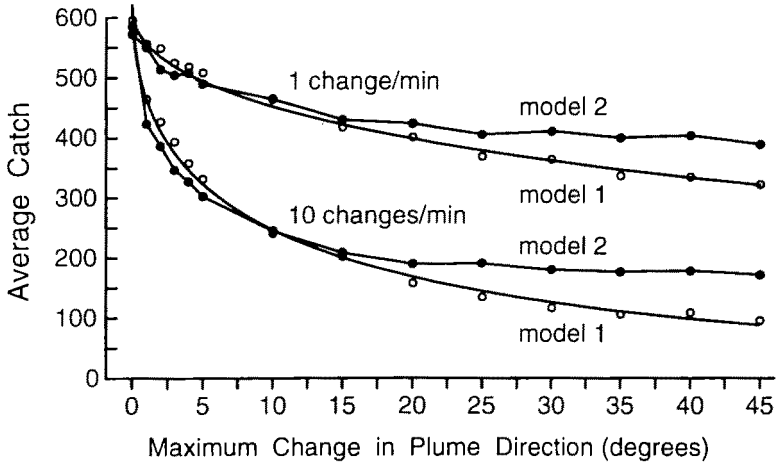


FIG. 10. Effect of maximum change at random in a random walk in plume direction (at 1 or 10 changes per minute at random) on the average catch over an hour of simulated time for model 1 and model 2 insects. Model 1 insects (those used in most simulations) moved forward in the current wind direction after losing the plume during a wind shift. Model 2 insects moved forward in the former wind direction after losing the plume during a wind shift (thus they must remember their previous ground path before casting). Simulations otherwise as in Figure 6.

random effect was probably due to histograms composed of fewer catches with a narrow plume during one period of time compared to larger catches with a wide plume at another time in the simulation (see Figure 4). At a smaller range of 10–20°, no effect of plume width changes could be found on arrival pattern, indicating that at even more narrow and probably more realistic ranges there also would be no affects on randomness.

Random walks in wind speed dramatically modified the arrival pattern to become clumped and nonrandom (Figures 8 and 9). The effects of wind speed fluctuations were most evident when the wind could vary above and below the flight speed. A wind speed of 0.2 m/sec was used as the minimum speed necessary for anemotaxis and corresponds to the finding of Nakamura and Kawasaki (1984) that the moth *Spodoptera litura* oriented poorly at low wind speeds. However, removal of this parameter from the model had little affect on the randomness of arrival.

Compared to moths, relatively less is known about bark beetle flight orientation to pheromone. In the confines of a wind tunnel, bark beetles fly erratically, which has discouraged more detailed studies (Choudhury and Kennedy, 1980; Salom and McLean, 1991a). In the field, bark beetles generally are recaptured downwind in the absence of attractive odors (Salom and McLean, 1991b), but this can be the result of either a directed flight downwind or a random drift with wind (Helland et al., 1984; as in the models here). Salom and McLean (1991b) found *Trypodendron lineatum* flew across wind to opposite sides of a clear-cut valley, although more were caught downwind. Bark beetles are believed to fly upwind to attractive odor sources based on direct observation and on catches of traps correlated with wind direction (references in Byers 1988; Salom and McLean, 1991b). Byers (1988) mounted wind vanes on traps to prove that western pine beetles, *Dendroctonus brevicomis*, fly upwind to pheromone.

Bark beetles can accumulate within a plume at higher wind speeds and appear to fly in groups as in the model. On May 19, 1984, in Denmark in a clear-cut about 1 km from the study site, I observed *Ips typographus* taking off from a brood log pile. Most flew downwind (one beetle at about 3.3 m/sec over 50 m as I ran down a road parallel to its flight path) while fewer beetles flew across the wind. Nearby, I noticed several tens of *Ips typographus* at the same time as they flew slowly upwind to a large fallen Norway spruce undergoing colonization. The beetles were orienting upwind presumably within a pheromone/host odor plume from at least as far as 50 m from the tree. The beetles were tossed horizontally across the wind line, or possibly were casting, as they slowly progressed upwind at a ground speed of at most 0.5 m/sec (compared to my slow walking) and often were blown downwind during gusts. The wind was unidirectional but variable in speed from about 1 to 4 m/sec (fan anemometer at 1.5 m for 20–30 sec). Most individuals were flying at 2–4 m height

(practically all <6 m) while 15–30 m from the tree, but generally lower (1–3 m) when approaching within 10 m of the tree.

Many moths have a zigzagging flight during anemotaxis (Kennedy, 1983; Baker and Haynes, 1987), while model insects flew directly upwind with random deviations. The programmed counterturning flight could be included for more realism but should not greatly affect the relationships since the mean direction of a zigzag is still directly upwind with random deviations. In spite of the simplifications of the model, simulated insects could reliably reach the odor source using only a combination of odor-modulated anemotaxis and casting. Improvements to the model with regard to plume dimensions, orientation and flight movement parameters, and wind effects probably will not alter the basic conclusion that fluctuations in wind speed and direction modify an otherwise random arrival pattern to one that is clumped and nonrandom. Most reports on arrival of insects to traps can not be analyzed for random arrival patterns as done here since the sampling periods were of hours or days (e.g., Byers and Löfqvist, 1989). However, Nakamura and Kawasaki (1984) report the mean wind velocity (every 5 min) with 1-m sampling periods of arrival of *S. litura* to pheromone traps over 2 hr, but they did not speculate about randomness of the arrivals. A chi-square analysis of the *S. litura* data (their Figure 1) shows that this moth exhibited a clumped arrival pattern (23 *df*, $P = 0.03$).

The simulation model is available from the author as a compiled program for IBM-compatible personal computers with VGA monitor (please send a formatted disk). The software also can be downloaded from the Internet (http://alyssum.stud.slu.se:8001/~johnb/software.html).

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ENANTIOSPECIFIC PHEROMONE PRODUCTION AND RESPONSE PROFILES FOR POPULATIONS OF PINE ENGRAVER, *Ips pini* (SAY) (COLEOPTERA: SCOLYTIDAE), IN BRITISH COLUMBIA

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Abstract—Analyses of the enantiomeric composition of ipsdienol produced by individual male pine engravers, *Ips pini* (Say), from six populations in British Columbia, support the hypothesis that New York and Idaho races of this species hybridize in southeastern British Columbia. Production profiles, expressed as frequency distributions of (+):(−) ipsdienol ratios [= ratio of (*S*)-(+)-ipsdienol to (*R*)-(−)-ipsdienol], were bimodal for four western British Columbia populations. The (+):(−) ratios ranged from 63:37 to 71:29, consistent with those previously found for the New York race. The profile for a southeastern population from Radium, British Columbia, was intermediate between those for the four western British Columbia populations and that from one population in Kimberley, British Columbia, just south of Radium. Males in the Kimberley population produce predominantly (*R*)-(−)-ipsdienol, typical of California and Idaho males. Response profiles of different individuals of *I. pini*, determined by captures of beetles in multiple-funnel traps baited with ipsdienol of 11 different (+):(−) ratios, were not consistent with production profiles. Populations in Williams Lake and Princeton, in western British Columbia, and Radium, in southeastern British Columbia, had response profiles with maximal attraction to ipsdienol over a broad range of (+):(−) ratios, falling off as enantiomeric purity was approached at either end of the

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spectrum. This type of response profile is consistent with that for the New York race, which has been shown to respond optimally to (+):(−) ratios ranging from 40:60 to 70:30. The response profile of the Kimberley population gradually declined from maximal attraction to ipsdienol with a (+):(−) ratio of 2:98 to the lowest response at a (+):(−) ratio of 98:2. The attraction of *I. pini* to chemical stimuli in California is interrupted by ipsdienol with a (+):(−) ratio >5:95, a pheromone of a host competitor, the California five-spined ips, *Ips paraconfusus* Lanier. We hypothesize that the Idaho race, which does not compete with *I. paraconfusus* due to geographical separation, is characterized by a Kimberley-type enantiomeric response profile, intermediate between those of the New York and California races.

Key Words—Ipsdienol, *Ips pini*, Scolytidae, aggregation pheromone, enantiomer, geographic variation, intrapopulation variation, speciation, competitive exclusion.

INTRODUCTION

The principal male-produced aggregation pheromone of the pine engraver, *Ips pini* (Say)(Coleoptera: Scolytidae), is ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol), a monoterpene alcohol with one asymmetric carbon center and two enantiomeric configurations (Seybold, 1993). Pioneering research on volatile extracts trapped from populations of *I. pini* disclosed that males from California (Stewart, 1975; Birch et al., 1980) and Idaho (Plummer et al., 1976) produce only (*R*)-(−)-ipsdienol, while males from New York produce a 65:35 blend of (*S*)-(+) and (*R*)-(−) enantiomers (Lanier et al., 1980).

Recently, pheromone production profiles were determined for populations of *I. pini* based on the enantiomeric ratios of ipsdienol produced by individual beetles (Slessor et al., 1985; Miller et al., 1989). Males in populations from Hat Creek, California, and Kimberley in southeastern British Columbia produce primarily (*R*)-(−)-ipsdienol, with mean (+):(−) ratios [= ratio of (*S*)-(+)-ipsdienol to (*R*)-(−)-ipsdienol] of 9:91 and 11:89, respectively. In each population, however, there are a few beetles that produce primarily the (+) enantiomer. Beetles from Newcomb, New York, are characterized by a bimodal frequency distribution, with respect to enantiomeric composition, with a mean (+):(−) ratio of 57:43. A population from Princeton in southwestern British Columbia is characterized by a similar, but slightly broader, bimodal enantiomeric production profile, with a mean (+):(−) ratio of 66:34.

Seybold et al. (1995a) determined enantiomeric compositions of ipsdienol in volatiles captured from logs infested by males from 35 populations of *I. pini*, representing the North American range of the species. These included one population from southwestern British Columbia and a second population from southeastern British Columbia, very close to the locations used by Miller et al. (1989), and several populations from Washington, Idaho, and Montana. Seybold et al.

(1995a) hypothesized that there are two, possibly three, geographically separate pheromone variants or races of *I. pini*. Males in the "California" race, with a range extending from Washington to Arizona and New Mexico, produce ipsdienol with (+):(−) ratios of 6:94 to 2:98. The eastern "New York" race is characterized by (+):(−) ratios of 68:32 to 44:56. This race ranges from southern Appalachia, north along the Atlantic coast, through the US Lake states, and across the boreal forest of Canada, extending south in British Columbia to the US border. The third "Idaho" race is located in southeastern British Columbia, Idaho, and Montana (and possibly further east, west, and south); its males produce ipsdienol with (+):(−) ratios ranging from 9:91 to 5:95. Seybold et al. (1995a) speculated that the Idaho race may represent a zone of hybridization between the New York and California races.

Responses to ipsdienol-baited traps by *I. pini* in California and New York appear to correspond to the enantiomeric composition of ipsdienol produced by the respective races. In California, *I. pini* are attracted by (*R*)-(−)-ipsdienol. The attraction is significantly interrupted by as little as 5% of the antipode (Birch et al., 1980; Seybold, 1992). In New York, the (+):(−) ratio of ipsdienol eliciting maximal attraction of *I. pini* ranges from 70:30 to 40:60, inclusively (Lanier et al., 1980; Teale and Lanier, 1991; Teale et al., 1994). The enantiospecific response to (*R*)-(−)-ipsdienol in California may be driven by natural selection arising from competition with the California fivespined ips, *Ips paraconfusus* Lanier. Both species breed in *Pinus ponderosae* Laws. and *P. jeffreyi* Grev. & Balf. (Wood, 1982). The reproductive success of individuals of both species is reduced when they infest the same host material (Birch, 1978; Light et al., 1983). Male *I. paraconfusus* produce predominately (*S*)-(+)-ipsdienol as one component of their aggregation pheromone blend (Seybold, 1992; Seybold et al., 1995b). Attraction of *I. paraconfusus* to its pheromone is interrupted by the (−) enantiomer, to which *I. pini* is attracted (Light and Birch, 1977; Birch et al., 1980; Seybold, 1992).

We report the results of experiments in which four additional populations in southern British Columbia were characterized by their ipsdienol enantiomeric production profiles. We also tested different individuals from two of these new populations, as well as those from Princeton and Kimberley, British Columbia, for their field response to ipsdienol with enantiomeric (+):(−) ratios ranging from 2:98 to 98:2. Populations of *I. pini* have been tested previously in California and New York for their responses to ipsdienol over a broad range of enantiomeric ratios (Seybold, 1992; Teale et al., 1994).

METHODS AND MATERIALS

Production Profiles. Bolts of lodgepole pine, *Pinus contorta* var. *latifolia* Engelm., infested with live broods of *I. pini*, were collected from Osprey Lake,

Pemberton, and Williams Lake, British Columbia, in 1986, and Radium, British Columbia, in 1987. Bolts were placed in rearing cages and beetles were collected after they emerged as mature adults. Using the gelatin-pill-capsule technique (Borden, 1967), adult males from each of the four populations were restrained on uninfested bolts of lodgepole pine collected near Princeton, British Columbia. They were allowed to bore into the bark and feed for 24–48 hr. Abdomens from individual males were removed and each was crushed in 150 μl of pentane containing racemic 3-octanol (4.1 ng/μl) as an internal standard. Extracts were analyzed by splitless, capillary gas chromatography using a Hewlett-Packard (HP) 5890 and a fused silica column (J&W Scientific, Folsom, California; 30 m × 0.25 mm ID, 0.25 μm DB-1), before and after derivatization to acetyl lactate diastereomers (Slessor et al., 1985). Only extracts containing > 10 ng of ipsdienol were derivatized. Retention times of ipsdienol and its two derivatives were determined with racemic ipsdienol obtained from Borregaard Fine Chemicals, A.S. (Sarpsborg, Norway), and enantiomeric assignments were made according to Slessor et al. (1985). The identities and integrities of ipsdienol acetyl lactate diastereomers were verified by mass spectrometry using splitless, capillary gas chromatography (HP 5985B).

Response Profiles. For experiment 1, racemic ipsdienol (chemical purity >95%) was obtained from Borregaard Fine Chemicals, A.S. Two enantiomeric mixtures of ipsdienol [(+):(−) ratios of 98:2 and 2:98, respectively] were obtained from E. K. Czyzewska (Department of Chemistry, Simon Fraser University, Burnaby, British Columbia). For experiments 2–4, three enantiomeric mixtures [(+):(−) ratios of 98:2, 50:50, and 2:98, respectively] (chemical purities, 93, 98, and 83%, respectively) were obtained from Phero Tech Inc. (Delta, British Columbia).

In experiment 1, each lure consisted of 10 Microcap disposable pipettes (2 μl) (Drummond Scientific Co., Broomall, Pennsylvania), each sealed at one end and filled with ipsdienol, and placed in a polyethylene, microcentrifuge tube (1.8 ml) (Evergreen Scientific, Los Angeles, California). The release of ipsdienol with different enantiomeric ratios was achieved by adjusting the relative proportion of tubes filled with the different mixtures. The release rate of ipsdienol from each lure was approximately 0.1 mg/day at 24°C (determined by weight loss).

In experiments 2–4, each lure consisted of a 10-cm-length of C-flex tubing (ID = 1.6 mm; OD = 2.4 mm) (Concept Inc., Clearwater Florida), filled with an ethanol solution of ipsdienol (80 mg/ml). Solutions were prepared for each of 11 different enantiomeric ratios of ipsdienol. The release rate of ipsdienol from each lure was approximately 0.6 mg/day at 24°C (determined by collection of volatiles on Porapak-Q).

In all experiments, replicates of 12 eight-unit, multiple-funnel traps (Lindgren, 1983) (Phero Tech Inc.) were set in grids of 3 × 4 in stands of lodgepole

pine. Replicate grids were placed at least 100 m apart, and traps were spaced 10–15 m apart within each replicate. Each trap was baited, and suspended such that the top of each trap was approximately 1.3–1.5 m above ground level. No trap was within 2 m of any tree.

Experiments 1–4 determined the effect of the enantiomeric composition of ipsdienol on the attraction of *I. pini* near Princeton, Williams Lake, Radium, and Kimberley, British Columbia, respectively. Ten replicates were set for experiments 1–3 during the period of May 22–June 9, 1986; July 5–September 6, 1987; and July 7–September 9, 1987, respectively. Eight replicates were set for experiment 4 during the period of July 13–September 6, 1989. The 12 treatments consisted of 11 different enantiomeric ratios of ipsdienol and a control, randomly assigned within each replicate. In experiment 1, the control traps did not have any lures while in experiments 2–4, the control traps were baited with C-flex tubing containing ethanol alone. All lures were replaced, and treatments rerandomized within each replicate, at intervals of 3 to 4 weeks. Trap catches were pooled at the conclusion of each experiment.

Statistical Analyses. Data were analyzed using the SAS statistical package ver. 5.0 (SAS Institute Inc., Cary, North Carolina). Quantities of ipsdienol from individual abdominal extracts from males were transformed by $\ln(Y + 1)$ and subjected to one-way analysis of variance (ANOVA). Scheffé's multiple comparison test was performed when $P < 0.05$. For each population, coefficients of variation (CV) were determined for quantity and enantiomeric composition of ipsdienol, normalized by $\ln(Y + 1)$ and $\arcsin \sqrt{Y}$, respectively (Schlyter and Birgersson, 1989). Distributions of the enantiomeric compositions of ipsdienol, including data sets for populations from Princeton and Kimberley, British Columbia (Miller et al., 1989), were compared by χ^2 tests.

Trap catch data, transformed by $\ln(Y + 1)$ to remove heteroscedasticity, were analyzed by three-way ANOVA using location, treatment, replicate nested within location, and the interaction between location and treatment as model factors. For each location, data were analyzed by two-way ANOVA using replicate and treatment as model factors, first with all treatments and then with only the ipsdienol treatments. Eleven and 10 orthogonal contrasts were conducted for the first and second ANOVA's for each location, respectively, comparing the control against each ipsdienol treatment separately in the first case and comparing the response to racemic ipsdienol against each of the remaining ipsdienol treatments separately in the second.

Sex-ratio data [transformed by $\arcsin(Y)$], for catches ≥ 10, were subjected to full-factorial two-way ANOVA. For each location, untransformed sex-ratio data (not means), for catches ≥ 10, were regressed against the enantiomeric composition of ipsdienol, using treatment as the only factor in a general linear model. Tests of homogeneity of slopes were performed by analysis of covariance (ANCOVA) using location, and the interaction between location and the co-

variate, enantiomeric composition of ipsdienol as model factors. One contrast was performed, comparing the slope for the population in Kimberley against the slope for the remaining populations. For each location, we determined the Pearson correlation coefficient between mean proportional responses to each of 11 enantiomeric ratios of ipsdienol and the associated mean proportion of males in populations producing the same enantiomeric ratio (±5%) as determined above.

RESULTS

Production Profiles. Male *I. pini* from the Pemberton population produced the lowest amounts of ipsdienol per male compared to those from the other three populations (Table 1). Pemberton males were the least vigorous of all populations with respect to rates of boring and feeding. Beetles from Radium were reared from logs with the thickest phloem, probably influencing their vigor and hence the high level of ipsdienol production (Table 1). The quantity of ipsdienol produced by individual males varied markedly within each population (Figure 1), with coefficients of variation ranging from 66.0 to 167.3% (Table 1). As found by Miller et al. (1989) in other populations of *I. pini*, most males contained low quantities of ipsdienol and relatively few contained large amounts.

Pronounced bimodal frequency distributions of the enantiomeric compositions of ipsdienol, previously found for populations from Princeton, British Columbia, and Newcomb, New York (Miller et al., 1989), were found in three additional British Columbia populations (Pemberton, Williams Lake, and Osprey Lake) (Figure 2). Males from all three of these populations produced more (*S*)-(+)-ipsdienol than (*R*)-(−)-ipsdienol with mean (+):(−) ratios ranging from 63:37 to 71:29 (Table 1). There were no significant differences among the distributions of enantiomeric compositions of ipsdienol from the four westernmost British Columbia populations (Pemberton, Princeton, Osprey Lake, and Williams Lake) (χ^2 test, $df = 27$, $P = 0.194$) (Figure 2). The distributions for these populations were significantly different from that for the population from Kimberley (χ^2 test, all $df = 9$, all $P < 0.001$). In contrast, males from Radium produced almost equal amounts of both enantiomers (Table 1). The enantiomeric production profile for these males was significantly different from the profiles for males from the Kimberley population (χ^2 test, $df = 9$, $P < 0.001$) and the four western British Columbia populations (χ^2 test, all $df = 9$, all $P < 0.001$) (Figure 2).

Response Profiles. Ips pini showed considerable variation in attraction to ipsdienol-baited multiple-funnel traps as a consequence of enantiomeric composition (Table 2). In all populations, significantly fewer beetles were caught in traps baited with an ipsdienol (+):(−) ratio of 98:2 than in traps baited with racemic ipsdienol (Figure 3). Significantly fewer beetles were caught in all traps

TABLE 1. QUANTITIES AND ENANTIOMERIC COMPOSITIONS OF IPSDIENOL PRODUCED BY INDIVIDUAL MALE *Ips pini* (SAY) FROM 4 LOCALITIES IN BRITISH COLUMBIA

	Quantity of ipsdienol (ng)			Enantiomeric ratio of ipsdienol (*S*)-(+):(*R*)-(−)				Correlations between quantity and enantiomeric composition[c]	
Locality	*N*	Mean ± SE[a]	CV (%)[b]	*N*	Mean ratio[c]	SE	CV (%)[d]	*r*	*P*
Pemberton	68	47 ± 15 a	167.3	14	65:35	4.9	30.4	−0.01	0.965
Williams Lake	75	158 ± 28 b	93.7	38	71:29	2.0	22.7	−0.02	0.896
Lake Osprey	62	168 ± 34 b	74.8	35	63:37	2.7	25.8	−0.05	0.769
Radium	139	197 ± 21 b	66.0	87	52:48	2.5	32.7	+0.18	0.105

[a] Means followed by the same letter are not significantly different at $P = 0.05$ [Scheffe's multiple comparison test on data transformed by $\ln(Y + 1)$].
[b] Data transformed by $\ln(Y + 1)$ prior to analysis.
[c] Only for extracts containing > 10 ng of ipsdienol.
[d] Data, expressed as proportion of (+) enantiomer in total amount of ipsdienol, transformed by arcsin $\sqrt{Y}$ prior to analysis.
[e] Pearson correlation coefficient adjusted for degrees of freedom.

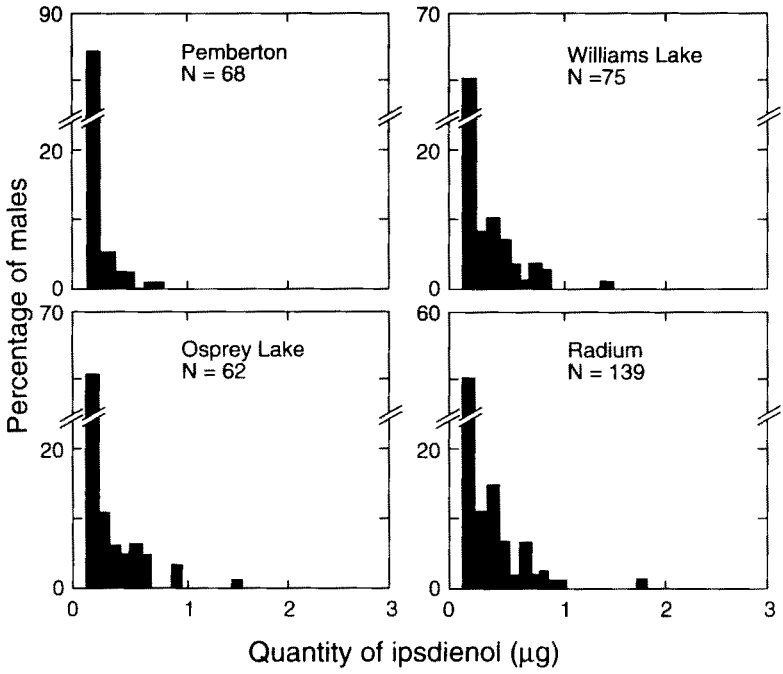

FIG. 1. Frequency distributions of the quantities of ipsdienol produced by individual male *Ips pini* from four populations in British Columbia.

baited with a (+):(−) ratio of 2:98 than in traps baited with racemic ipsdienol in Williams Lake, Radium, and Princeton. Except for one contrast, significantly more beetles were caught in traps baited with ipsdienol than in control traps (orthogonal contrasts, ANOVA, all $P < 0.029$). The exception was in Kimberley, where there was a weakly significant increase of catches in traps baited with a (+):(−) ipsdienol ratio of 98:2 relative to those in traps baited with ethanol alone (orthogonal contrast, ANOVA, $P = 0.079$.

The enantiospecific response profiles varied significantly between locations ($P < 0.001$; Table 2). The most obvious differences were related to the attraction of *I. pini* to (+):(−) ipsdienol ratios of 2:98 and 10:90. For both ratios, catches were low in Williams Lake and Princeton but high in Kimberley (Figure 3). In Radium, catches to traps baited with (+):(−) ratio of 10:90 were high but low to those baited with a (+):(−) ratio of 2:98.

The sex ratio of captured *I. pini* varied among locations and enantiomeric treatments (Table 2). As in California (Seybold, 1992), the proportion of males caught in ipsdienol-baited traps increased as the proportion of (*S*)-(+)-ipsdienol

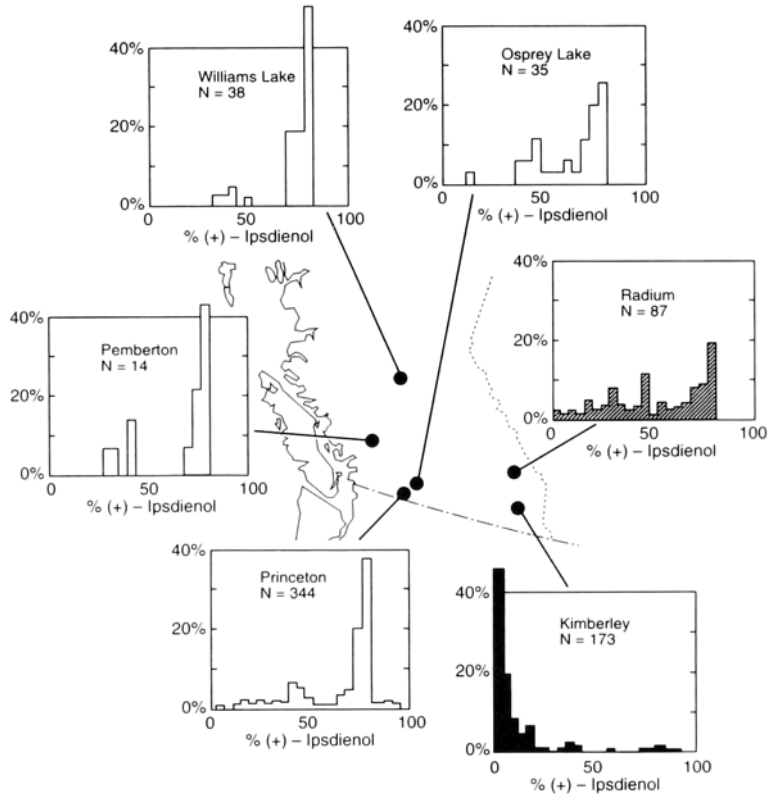

FIG. 2. Frequency distributions of the enantiomeric compositions of ipsdienol produced by individual male *Ips pini* from six populations in British Columbia. Data for Princeton and Kimberley are reproduced from Miller et al. (1989). Histograms with different shading patterns are significantly different (see text).

increased (Figure 4). The slope of the regression line for the Kimberley population was significantly different from that of the other three populations (orthogonal contrast, ANCOVA, $P = 0.003$). Accordingly, the effect of the interaction between location and enantiomeric composition of ipsdienol was not significant when the Kimberley population was omitted from the analysis. When Kimberley was included, the effect was significant ($P = 0.021$; Table 2).

The association between enantiospecific production and response profiles differed between locations. In Radium and Princeton the correlations between mean (+):(−) ipsdienol ratios produced by males within the population and the respective enantiospecific response profile were significantly different from zero

TABLE 2. ANALYSIS OF VARIANCE OF EFFECTS OF LOCATION (WILLIAMS LAKE, RADIUM, PRINCETON, AND KIMBERLEY, BRITISH COLUMBIA], ENANTIOMERIC COMPOSITION OF IPSDIENOL [11 DIFFERENT (+):(−) RATIOS], INTERACTION BETWEEN LOCATION AND ENANTIOMERIC COMPOSITION, AND REPLICATE (N = 8 OR 10) NESTED WITHIN LOCATION ON NUMBER AND SEX RATIO OF *Ips pini* (SAY) CAPTURED IN IPSDIENOL-BAITED MULTIPLE-FUNNEL TRAPS

Source	Trap catch[a]			Proportion of males[b]		
	df	*F*	*P*	*df*	*F*	*P*
Location (*A*)	3	86.95	<0.001	3	309.81	<0.001
Enantiomeric composition (*B*)	10	33.98	<0.001	10	11.67	<0.001
Replicate nested within location (*C*)	34	15.81	<0.001	34	4.45	<0.001
A * *B*	30	5.77	<0.001	28	1.68	0.021
Error	319			260		

[a]Data transformed by $\ln(Y + 1)$ prior to analysis.
[b]Data transformed by $\arcsin(Y)$ prior to analysis.

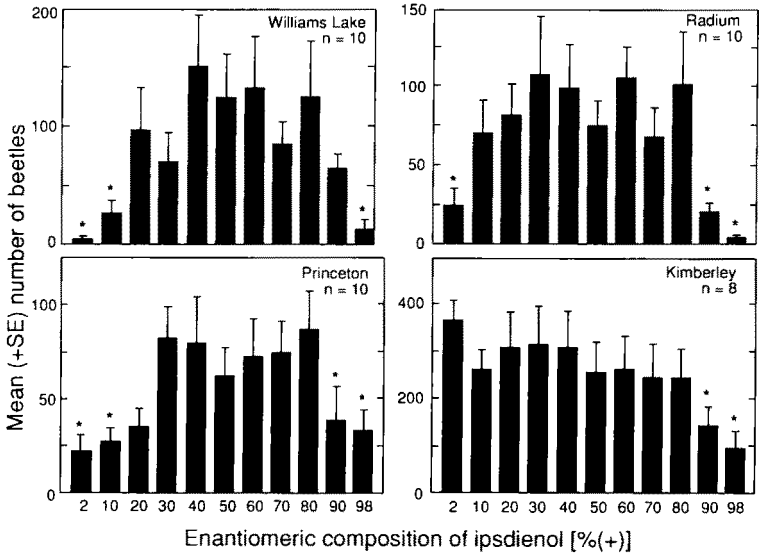

FIG. 3. Effect of the enantiomeric composition of ipsdienol on the capture of *Ips pini* in multiple-funnel traps at four locations in British Columbia. Asterisks indicate significant difference in trap catches relative to those in traps baited with racemic ipsdienol, orthogonal contrasts, $P < 0.08$.

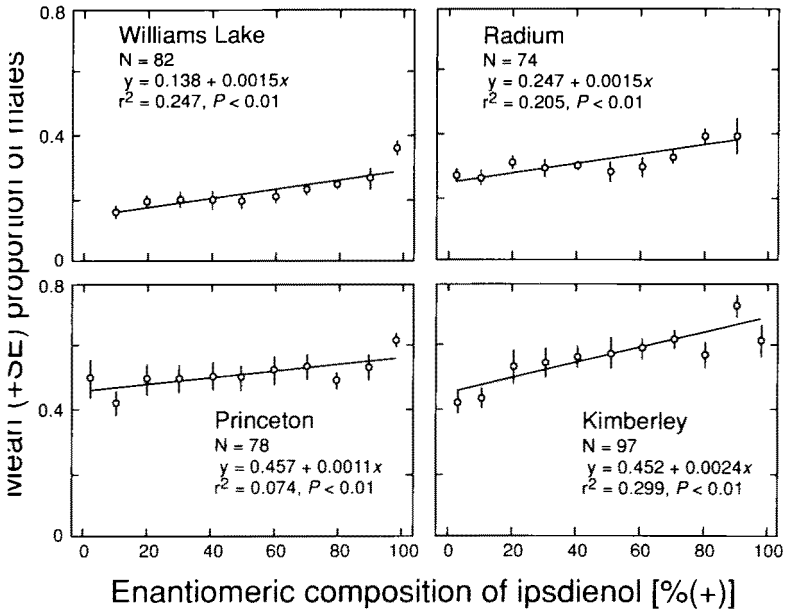

FIG. 4. Effect of the enantiomeric composition of ipsdienol on the sex ratio of *Ips pini* captured in multiple-funnel traps at four locations in British Columbia. Slope of the regression line for Kimberley is significantly different from those for the other three populations (see text).

(Table 3). The correlation was only weakly significant for the population from Kimberley and not significant for Williams Lake. In no case did the variation in one variable explain more than 50% of the variation in the other variable (all $r^2 < 0.475$).

DISCUSSION

The similarity of enantiospecific ipsdienol production profiles for males from the four western-most British Columbia populations examined (Figure 2) to that previously examined from Newcomb, New York (Miller et al., 1989) supports the hypothesis that the New York race ranges across the boreal forest in Canada and extends southward into British Columbia (Seybold et al., 1995a). The similarity of response profiles for populations from Williams Lake and Princeton to that for a population from Syracuse, New York (Teale et al., 1994) further supports this hypothesis, although the distribution was narrower for the

TABLE 3. CORRELATIONS BETWEEN ENANTIOSPECIFIC IPSDIENOL PRODUCTION PROFILES FOR MALE *Ips sini* (SAY) AND ENANTIOSPECIFIC RESPONSE PROFILES OF MALE AND FEMALE *I. pini* IN 4 LOCALITIES IN BRITISH COLUMBIA

Locality	Pearson correlation coefficient (r)	P
Williams Lake	0.356	0.283
Kimberley	0.538	0.088
Princeton	0.624	0.040
Radium	0.689	0.019

Syracuse population. The dissimilarity of the response profile for the Kimberley population to those for the other three British Columbia populations (Figure 2) is consistent with dissimilarities in production profiles previously reported between Kimberley and Princeton populations (Miller et al., 1989). The Kimberley population has previously been identified as part of the Idaho race (Seybold et al., 1995a). In our study, this is the only British Columbia population in which *I. pini* showed a considerable attraction to ipsdienol with a (+):(−) ratio of 2:98 (Figure 2), a preferred ratio for the California race (Birch et al., 1980; Seybold, 1992).

The population from Radium showed characteristics suggesting a combination of traits of the New York race and the Idaho or California races. The enantiospecific production profile was broad (Figure 1), with the highest coefficient of variation (Table 1). The Radium profile lacked both bimodality, common to the New York race, and a sharp peak at a (+):(−) ratio of 2:98, common to the Idaho and California races (Miller et al., 1989; Seybold et al., 1995a). The response profile for the Radium population was also broader than for populations from Williams Lake and Princeton (Figure 3), showing an attraction to a (+):(−) ratio of 10:90. However, beetles in the Radium population were not highly attracted to a (+):(−) ratio of 2:98, as was evident in the Kimberley population. These data suggest that the Radium population lies within an intergradation zone of the New York race and the Idaho or California races.

Our results demonstrated significant variation in the attraction of *I. pini* to ipsdienol for all four populations in British Columbia (Figure 3). In no case did we find evidence of a narrow range of enantiospecific responses to ipsdienol as shown by *I. pini* in California (Birch et al., 1980; Seybold, 1992). The intrapopulational variation in attraction of *I. pini*, with respect to variation in enantiomeric composition of ipsdienol, should reflect the relative benefits gained by the responding individuals. The broad range of equal levels of attraction in all British Columbia populations examined (Figure 3) should, therefore, reflect

similar fitnesses in the responding individuals. However, initial preferences shown by individuals may not be associated with subsequent preferences by the same individuals. Cardé et al. (1976) showed that initial preferences by individual Oriental fruit moths, *Grapholita molesta* (Busk), were not associated with their subsequent preferences, yet the response profiles for the population were the same on both occasions. Such probability functions may be determined, in part, by age and vigor, as well as by genetics. The benefits may accrue from the average of repeated choices among individuals rather than specific choices.

The enantiospecific responses with respect to sex ratio (Figure 4) should reflect differential benefits to males and females. In Kimberley, females showed the strongest preference for ipsdienol when proportions of the (+) enantiomer were lowest. This trend is consistent with the production of predominantly (*R*)-(−)-ipsdienol by males (Figure 2) and the declining profile of total response in the population as the proportion of (*S*)-(+)-ipsdienol increased in the bait stimulus (Figure 3). However, it is not clear why this trend, *albeit* at a lower intensity, should be evident in the other three populations. It is possible that it is a residual sex-linked trait from other selection pressures or, possibly, a trait that cannot vary in a nonlinear fashion due to genetic constraints.

Our results show that the population profile for the response of bark beetles to a pheromone signal may not be predicted necessarily by the population profile for the production of the signal. The broad response profiles for beetles in Kimberley and Williams Lake differed from their narrow production profiles (Figures 2 and 3). The correlations between production and response were not strongly significant in either location (Table 3). In Princeton and Radium, only 39% and 47% of the variation in response was explained by the variation in production (Table 3).

We can only speculate on the composition underlying the response profiles. Populations may consist of generalists with the population response profile equating to the probability profile for response by an individual beetle. Alternatively, populations may have structure through a diverse collection of individuals, each with a narrow response range. Our correlations of population profiles are not necessarily indicative of genetic correlations within individuals.

Our results suggest that pheromone-mediated behavioral separation may not be necessarily a premating isolation mechanism leading to speciation. Two distinct strains of the European corn borer, *Ostrinia nubilalis* (Hübner), occur in Europe and North America, differing in the relative use of (*E*)- and (*Z*)-11-tetradecenyl acetates as pheromones (Kochansky et al., 1975; Klun and Cooperators, 1975; Anglade et al., 1984; Barbattini et al., 1985; Peña et al., 1988; Löfstedt, 1990). The two strains hybridize readily where they are sympatric (Roelofs et al., 1985; Cardé, 1986; Klun and Huettel, 1988). In a similar fashion, *I. pini* in the population from Kimberley showed a lack of enantio-

specificity to ipsdienol even though males in the population were very enantiospecific in the production of ipsdienol. The population from Radium provides evidence of hybridization between two, possibly three, races of *I. pini*, presumbly due to plasticity in behavioral responses.

A complete understanding of the use of pheromones by *I. pini* should also consider the effect of a second pheromone, lanierone (Teale et al., 1991). Attraction of males and females to ipsdienol is synergized by lanierone in New York, Wisconsin, Montana, and British Columbia, yet is unaffected in California (Seybold et al., 1992; Miller et al., 1996).

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SOCIAL CLOSURE, AGGRESSIVE BEHAVIOR, AND CUTICULAR HYDROCARBON PROFILES IN THE POLYDOMOUS ANT *Cataglyphis iberica* (HYMENOPTERA, FORMICIDAE)

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Abstract—Nestmate recognition was studied in the polydomous ant *Cataglyphis iberica* (Formicinae) in the laboratory. The study examined six colonies collected from two different populations 600 km apart in the Iberian peninsula (Barcelona and Murcia). Introduction of an alien worker into an allocolonial arena always ended in death to the intruder, demonstrating that in this species societies are extremely closed. Dyadic encounters composed of individuals from different colonies in a neutral arena confirmed the existence of high aggression between allocolonial individuals. We also investigated variability in the composition of the major cuticular hydrocarbons between the colonies used in the behaviorial tests. There were marked quantitative differences between the profiles of ants from the two populations, suggesting that the populations are completely segregated. Cuticular profiles within a population tended to be more similar, but were nevertheless colony specific. The degree of colony closure in *C. iberica* seemed to be independent

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of geographic distance since aggression between the colonies was always at its maximum, irrespective of their population origin.

Key Words—Ants, Formicinae, polydomy, nestmate recognition, aggression, cuticular hydrocarbons, colony closure, *Cataglyphis iberica*.

INTRODUCTION

Social organization in ants is based on, among other things, colony closure that preserves its social integrity and prevents intrusion by individuals alien to the society. This necessitates a capacity to recognize and discriminate between familiar and unfamiliar individuals. Such discrimination generally induces aggression towards the unfamiliar intruder. As early as 1904, Fielde (1904) hypothesized a chemical role in recognition. Howse (1975) suggested that this discrimination ability was based essentially on a chemical signature that each member of the colony carries on its cuticle, a hypothesis that was subsequently confirmed by experimental work (reviewed by Howard, 1993; Lorenzi et al., 1996). Experiments in which alien cuticular extracts applied to workers elicited aggression towards them by their nestmates lent credence to the role of cuticular lipids in colony recognition (Bonavita-Cougourdan et al., 1987; Nowbahari et al., 1990; Henderson et al., 1990). Nestmate recognition cues can be determined genetically (Vander Meer et al., 1985; Ross et al., 1987; Provost, 1991), but may also include environmental substances that blend into the colony odor (Jutsum et al., 1979; Obin, 1986; Obin and Vander Meer, 1988). Congruency was found between the hydrocarbon composition of postpharyngeal gland contents and those of the cuticular washes (Bagnères and Morgan, 1991; Do Nascimento et al., 1993). Recent studies pointed to the role of the postpharyngeal gland in storing and mixing the recognition chemicals and reapplying them on the cuticle, creating a unified colony odor (Soroker et al., 1994, 1995).

Earlier studies with *Cataglyphis cursor* have shown a correlation between intercolonial aggression and the geographic distance between colonies (Nowbahari and Lenoir, 1984; Nowbahari et al., 1990). In this species, whose colonies multiply by fission, allocolonial workers that originate from neighboring nests are readily accepted, whereas workers originating from distant colonies are always rejected.

Cataglyphis iberica is a monogynous and polydomous species that is limited to the Iberian peninsula (Tinaut and Plaza, 1989; Cerdá and Retana, 1992). Each colony is composed of several satellite nests, between which there is an exchange of individuals through adult transport. Its biology is typical of the majority of species in this genus: it is a diurnal scavenger ant species that is active during the hottest period of the day (Cerdá, 1988; Cerdá and Retana, 1988).

In this study, we tested colonial closure in *C. iberica* and compared intercolonial aggression in relation to divergence in their cuticular hydrocarbon profiles and the geographic distances separating the colonies.

METHODS AND MATERIALS

Origin and Rearing of Colonies. This study was conducted with six colonies collected from two different populations in Spain, about 600 km apart. Three colonies were collected near Barcelona (population B): colony B1, collected from Bellaterra, was comprised of three satellite nests containing a total of 1650 workers. Colonies B2 and B3 were both collected from Sant Cugat (3 km away from Bellaterra), each of which was comprised of two satellite nests containing a total of 1918 and 975 workers, respectively. Three additional colonies were collected in the province of Murcia (population M). Colony M1 was from La Paloma (near Murcia) and included two satellite nests with a total of 1260 workers. Colonies M2 and M3 were collected from Cabo de Gata (about 200 km South from La Paloma) each composed of a single nest containing 450 and 325 workers, respectively.

In the laboratory, the colonies were reared in plastic boxes (11.5 × 8.0 × 2.5 cm) in which the interior relative humidity was maintained at 50%. Each satellite nest occupied one box, but all satellites belonging to the same colony were connected by plastic tubes to a common foraging arena (35 × 35 cm). As the species is very thermophilic (Cerdá and Retana, 1988), the arena was heated to 25 ± 2°C by a lamp to allow adequate temperatures for foraging activity. Food (meal worms, cricket larvae, honey-apple mixture) was provided three times a week and water *ad libitum*. The nests were maintained at 10:14 D:L photoperiod.

Introduction of Workers into an Allocolonial Arena. For each test one worker was introduced into an allocolonial foraging arena that was lined with filter paper. Tests were performed with at least a 1-hr break between replicates, and the filter paper was changed for each replicate. The behavioral interactions with the resident ants were observed while measuring two time variables: the latency before the first attack (first attack time), and the time it took to kill the introduced worker (death time). The number of resident workers that were present in the arena was monitored at the beginning of each test. All tests were conducted between 10:00 and 16:00 hr during the daily activity period of the ants when foragers were present in the arena. Each test lasted until the introduced worker was killed. The tests conducted included all possible combination of the six colonies: B × B (43 tests), M × M (52 tests), and B × M (22 tests).

Dyadic Encounters. Tests of internidal aggression consisted of encounters between two alien ants in a neutral arena made up of a circular plastic box (8

cm in diameter) coated on the inner walls with Fluon. Before each test the arena was thoroughly cleaned with alcohol. Each encounter lasted for 10 min and involved individuals originating from different colonies that were either taken from the same population (type I encounters, six combinations) or taken from different populations (type II encounters, nine combinations) (see Table I below). Initially, five replicates were performed for each combination, which involved different individuals in order to avoid familiarization or cross-contamination by cuticular lipids. The behavior of only one of the contestants (alternatively selected) was recorded in each test, and aggression was calculated as relative frequencies of aggressive acts out of the total interactions observed during the encounter. The following behaviors were considered as aggression: opening of the mandibles, biting, or flexing the gaster. On the other hand, mutual investigation and submissive posture were considered to be neutral or amicable acts. In a few of the tests (~ 12%) there were neither contacts nor interactions between the ants, and the ants displayed mutual avoidance. Such indifference is also often observed in the field. These cases were excluded from further calculations to avoid an artificial lowering of the magnitude of aggression and therefore bias the statistics.

Chemical Analysis. For the chemical analyses, postpharyngeal glands of 30 ants from each population were dissected under distilled water and immediately immersed in 100 μl of pentane for extraction. The pooled samples were analyzed by coupled gas chromatography–mass spectrometry (Finnigan quadrupole or Fisons MD800 quadrupole) in the EI and CI (using methane as reagent gas) modes. The samples were run on an HP-5 fused silica capillary column that was heated from 150°C to 250°C at a rate of 5°C/min, or alternatively on a DB-5 fused silica capillary column that was temperature programmed from 120°C to 300°C at 3°C/min with an initial hold of 3 min. The various hydrocarbons were identified by their fragmentation patterns as compared to published spectra (Nowbahari et al., 1990; Bagnères et al., 1991).

The contents of the postpharyngeal and cuticular washes were compared by gas chromatography using the elution pattern and including reference compounds.

To compare cuticular hydrocarbon profiles between colonies used in the behavioral studies, cuticular washes from individuals were analyzed by gas chromatography. Six individuals were taken at random from each of the colonies and individually analyzed: each ant was washed for 10 min in 1 ml of acetone. The solution was then dried by nitrogen and the residue was redissolved in 30 μl acetone, of which 2 μl were injected into a gas chromatograph (Delsi 300) equipped with a capillary column (Chrompak CPSIL 5 WCOT 25 m × 0.22 mm in diameter), temperature programmed from 100°C to 280°C at a rate of 3°C/min). Peak integration was accomplished with an ENICA 21 integrator.

Calculations and Statistical Methods. All calculations and comparisons

were made by using the mean ± SE. The α value for all the statistical tests used (discriminant analysis, Fisher's PLSD, χ^2, Mann-Whitney, and Pearson's) was less than 5%. The comparison of cuticular profiles of individuals from the studied colonies was performed by a discriminant analysis (Statistica for Windows) based on the compounds with a relative intensity above 1% in at least one of the samples (a total of 33 compounds).

RESULTS

Aggression Towards Ants Introduced into Allocolonial Arena. All ants introduced into an allocolonial arena were eliminated without a single case of adoption. The resident ants in the arena were very aggressive towards the alien intruders and engaged in mortal attack as soon as the intruder ant was detected in the arena. The average latency to the first attack was 19.1 ± 3.3 sec, and the average time that elapsed until the alien ant was killed was 17.0 ± 2.3 min. Significant relationships were found between the two variables (the first attack time and the death time) and the number of foragers present in the arena (Pearson's $r = 0.25$, $P = 0.007$ in both cases), but the low r^2 (=0.06) value indicated that the level of aggressive response was only weakly dependent on the number of resident foragers in the arena.

In addition, the geographic distance between the colonies did not influence the latency of the first attack (Pearson's $r = 0.11$, NS), but geographic distance was weakly but significantly correlated with death time ($r = 0.37$, $P < 0.001$). The greater the geographic distance between the tested colonies, the quicker the alien ants were eliminated.

Aggression during Dyadic Encounters. In eight of the 75 dyadic encounters conducted there were no interactions between ants. These ants neither moved nor was there was any contact between them. These encounters were therefore excluded from further calculations in order to avoid any artificial reduction in the mean aggressive response. In the 67 encounters taken for analysis, workers of *C. iberica* always engaged in aggressive interactions after a short antennal contact, and rarely manifested neutral or amicable behavior. This is reflected in the high value of the frequency of aggressive acts calculated for all the encounters (0.70 ± 0.03).

The frequency of aggressive acts was rather homogeneous between the various combinations, ranging from 0.48 for the encounters involving ants from colonies B3 and M1 to 0.87 for the encounters between ants from colonies B2 and B3. When tests are grouped as type I encounters (workers originating from different colonies from the same population) and type II encounters (workers originating from colonies from different populations), no differences were found between the mean values of aggressive frequencies for each type of dyadic encounter (Table 1, U Mann-Whitney test, $P > 0.1$).

TABLE 1. AGGRESSIVE FREQUENCIES (MEAN ± SE) FOR TYPE I AND II ENCOUNTERS[a]

Dyadic tests	Aggression frequency
Type I tests	
B1-B2(5)	0.69 ± 0.11
B1-B3(5)	0.63 ± 0.14
B2-B3(5)	0.87 ± 0.08
B-B(15)	0.73 ± 0.07[a]
M1-M2(4)	0.71 ± 0.12
M1-M3(3)	0.54 ± 0.12
M2-M3(5)	0.72 ± 0.14
M-M(12)	0.67 ± 0.07[a]
Type II tests	
B1-M1(5)	0.58 ± 0.17
B1-M2(5)	0.73 ± 0.08
B1-M3(4)	0.85 ± 0.10
B2-M1(5)	0.73 ± 0.08
B2-M2(5)	0.70 ± 0.12
B2-M3(4)	0.86 ± 0.07
B3-M1(4)	0.48 ± 0.15
B3-M2(4)	0.76 ± 0.09
B3-M3(4)	0.65 ± 0.08
B-M(40)	0.70 ± 0.04[a]

[a] No differences were found between intrapopulation mean values (Fisher's PLSD, $P > 0.1$).

We further tested whether the levels of intercolonial aggression within a population differed from that between populations. The frequency of aggressive acts in the tests within a population (0.73 ± 0.07 and 0.67 ± 0.07 for populations B and M, respectively) was as high as the frequencies in the tests between populations (0.70 ± 0.04) (Table 1, Fisher's PLSD, $P > 0.10$).

An examination of the workers after an hour of a dyadic encounter showed that despite the high aggression between the ants, the dyadic interactions rarely ended in death (18 deaths of 134 tested workers) or injury (24 injured of 134) to one or both ants. The number of injured and killed workers was not different between type I and type II encounters ($\chi^2 = 0.064$, $P = 0.8$).

Chemical Analysis. The postpharyngeal gland exudates of ants from the two populations contained almost exclusively saturated hydrocarbons, including *n*-alkanes and mono- and dimethyl alkanes ranging from C_{12} to C_{34}. There was an additional series of monomethyl and dimethyl *n*-alkanes of higher molecular weight, but the intensities of the peaks did not allow proper identification (Table 2). The hydrocarbon profiles from ants belonging to populations B and M were qualitatively similar but differed quantitatively. The dominant compounds in the

TABLE 2. COMPOUNDS IDENTIFIED FROM POSTPHARYNGEAL GLANDS DISSECTED FROM INDIVIDUALS BELONGING TO NESTS COLLECTED FROM BARCELONA OR MURCIA POPULATIONS[a]

Compounds[b]	Relative amount		Compounds	Relative amount	
	Barcelona	Murcia		Barcelona	Murcia
Dodecane	t	t	4 m C_{24}	t	t
Tridecane	t	t	C_{25}	t	t
11 m C_{13}	t	t	11 m C_{25}	t	t
Pentadecane	t	t	7 m C_{25}	t	t
Hexadecane	t	t	5 m C_{25}	t	t
6 m C_{16}?	t	t	11,15 dm C_{25}	t	t
4 m C_{16}	t	t	3 m C_{25}	t	t
C_{17}	t	t	5,9 dm C_{25}	t	t
8,12 dm C_{16}	t	t	x,12 dm C_{25}	t	t
5 m C_{17}	t	t	C_{26}	t	t
4 m C_{17}	t	t	11 + 13 + 15 m C_{26}	t	t
3 m C_{17}	t	t	6 m C_{26}	t	t
C_{18}	t	t	5 m C_{26}	t	t
8,12 dm C_{18}	t	t	4 m C_{26}	t	t
C_{19}	t	t	5,9 + 5,13 cm C_{26}	t	t
C_{20}	t	t	5,11 dm C_{26}	t	t
Oleic acid	t	t	4,6 dm C_{26}	t	t
C_{22}	t	t	4,10 + 4,12 dm C_{26}	t	t
11 m C_{23}	t	t	C_{27}	t	2.88 ± 2.72
10 m C_{23}	t	t	11 + 13 m C_{27}	t	5.19 ± 1.37
5 m C_{23}	t	t	7 m C_{27}	t	t
3 m C_{23}	t	t	5 m C_{27}	t	t
C_{24}	t	t	11,15 dm C_{27}	t	t
6 m C_{24}	t	t	16,18 dm C_{27}	t	t
5 m C_{24}	t	t	7,11 dm C_{27}	t	t

TABLE 2. CONTINUED

Compounds[b]	Relative amount		Compounds	Relative amount	
	Barcelona	Murcia		Barcelona	Murcia
3 m C_{27}	t	4.34 ± 0.62	*x,y* dm C_{29}	t	t
5,9 dm C_{27}	t	t	3,9 dm C_{29}	4.08 ± 1.04	2.32 ± 0.54
5,13 dm C_{27}	t	t	C_{30}	t	t
C_{28}?	2.02 ± 1.71	1.04 ± 1.01	6 m C_{30}	2.41 ± 1.15	1.92 ± 1.31
12 m C_{28}	t	6.21 ± 2.40	5 m C_{30}	t	t
11 + 13 + 15 m C_{28}	t	2.88 ± 0.31	4 m C_{30}	2.59 ± 0.64	1.39 ± 0.35
3,7 dm C_{27}	t	t	9,15 dm C_{30}	t	t
mix of monomethyl C_{28}	t	t	*x*, 8 dm C_{30}	t	t
			4,8 dm C_{30}	t	t
6 m C_{28}	t	t	6,14 + 4,14 dm C_{30}	t	t
5 m C_{28}	t	t	4,10 dm C_{30}	t	t
4 m C_{28}	t	1.19 ± 0.49	5,9 + 5,11 + 5,14 dm C_{30}	t	t
3 m C_{28}	t	t	C_{31}	4.76 ± 2.68	t
5,11 dm C_{28}	t	t	15 m C_{31}	3.06 ± 1.04	3.71 ± 0.62
4,8 dm C_{28}	t	t	9 m C_{31}	2.86 ± 0.38	t
C_{29}	5.87 ± 3.73	7.51 ± 6.46	7 m C_{31}	2.79 ± 0.83	t
3,7 + 3,9 dm C_{28}	t	t	5 m C_{31}	2.66 ± 1.04	t
11 + 13 + 15 m C_{29}	3.42 ± 1.08	15.19 ± 2.24	11,15 dm C_{31}	8.29 ± 2.35	2.64 ± 0.82
9 m C_{29}	1.07 ± 0.40	3.38 ± 0.50	11,17 + 12,16 dm C_{31}	1.37 ± 0.39	t
7 m C_{29}	1.31 ± 0.31	1.06 ± 0.89	7,15 dm C_{31}	1.07 ± 0.79	t
5 m C_{29}	4.42 ± 1.59	3.73 ± 0.78	3 + 4 + 5 m C_{32}	t	t
11,13 dm C_{29}	1.27 ± 0.55	7.24 ± 0.88	7,11 dm C_{32}?	1.82 ± 0.39	t
7,17 dm C_{29}	1.37 ± 0.73	t	C_{33}?	t	t
7,11 dm C_{29}	t	t	12, *x* dm C_{32}	1.18 ± 0.54	t
3 m C_{29}	9.82 ± 3.71	5.95 ± 1.67	4,22 dm C_{32}	3.00 ± 0.83	t
5,9 dm C_{29}	1.42 ± 0.45	1.84 ± 0.33	*x*,15 dm C_{32}	1.96 ± 1.24	t

mix of dm C_{32}	1.67 ± 0.60	t	12 m C_{33}	t	t
x,16 dm C_{32}	t	t	13,17 dm C_{33}	2.13 ± 0.51	t
6,30 dm C_{32}	t	t			

[a]Compounds with a relative amount that was less than 1% were marked as traces (t). The identity of compounds followed by a question mark is uncertain. The mean values (±SD, $N = 9$) given in the table are calculated from the cuticular hydrocarbon profiles of individuals used in the discriminant analysis.
[b]m = methyl; dm = dimethyl.

ants from both populations were nonacosane and a series of monomethyl and dimethyl nonacosanes. Ants from the Murcia population were distinguishable from those from the Barcelona population by having a larger proportion of heptacosane and octacosane compounds, notably C_{27} and its 13-, 11-, and 3-methyl branched derivatives, 12-methyl C_{28} and a mixture of 11- + 13- + 15-methyl C_{28}. These were present only in traces in individuals from population B. Finally, Barcelona ants were distinguishable by having a larger proportion of hydrocarbons with heavier molecular weight corresponding to hentriacontane, a series of monomethyl and dimethyl hentriacontane, and dimethyl dotriacontane.

Cuticular hydrocarbon profiles of the 36 ants from the six colonies were analyzed by discriminant analysis (Figure 1). A plot of the first and the second variables shows that individuals belonging to the same population clustered together forming two clearly separated groups. These two groups were separated mostly by the first discriminant variable. Within each population, individual grouping according to colony origin is apparent, and colonies were well separated by the second discriminant variable. The compounds that had an important weight in the analysis were heptacosane and a mixture of its 11-, 13-methyl-branched derivatives and 12-methyl C_{28}. It is worth noting that colony M1, which clustered distantly from M2 and M3 based on the hydrocarbon profile,

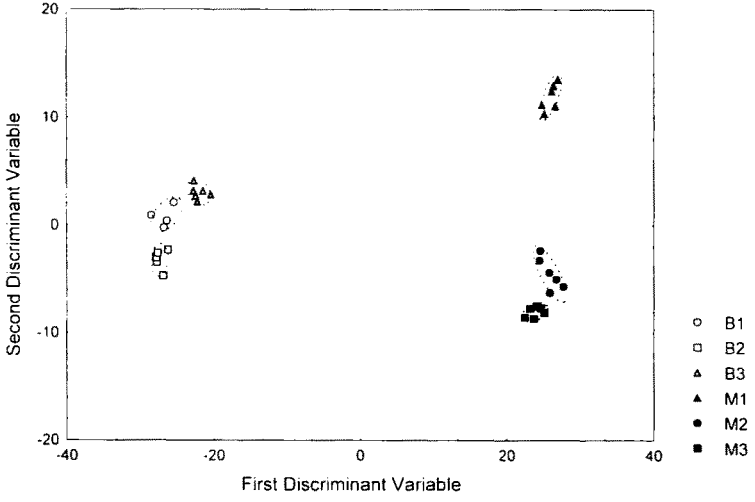

FIG. 1. A plot of the first two principal variables in the discriminant analysis of cuticular hydrocarbons of the six colonies of *Cataglyphis iberica*. Each colony group is surrounded by the confidence ellipse ($P < 0.05$).

was also geographically separated by 200 km from these two colonies (which were collected from the same locality).

We also conducted a discriminant analysis within each population on the basis of the same peaks. The results confirmed those shown in Figure 1. For the 18 ants analyzed on the basis of their cuticular profile within each population, individuals clearly clustered together according to their colony origin. Within population B, the compounds that had an important discriminant impact were a mixture of 15-, 13-, 11-, and 5-methyl C_{29} and 5,9-dimethyl C_{29}; while for population M, the discriminant compounds were 3-methyl C_{27}, 4-methyl C_{28} and 7-methyl C_{29}. This indicates that profile disparities between populations (B–M) and within populations (B–B or M–M) are not due to the same compounds.

DISCUSSION

Polydomous ant species are generally polygynous, with a low nestmate recognition system that seems to be the consequence of the low intracolonial relatedness (Hölldobler and Wilson, 1977; Breed and Bennett, 1987; Bourke and Franks, 1995). This low nestmate recognition system was observed, for example, in *Linepithema humile* (*Iridomyrmex humilis*) (Markin, 1968; Benois, 1973; Keller and Passera, 1989), *Amblyopone pallipes* (Traniello, 1982), and *Myrmica incompleta* (Lenoir et al., 1989). In *C. iberica*, societies are polydomous, but with a single queen per colony. Our results show that these colonies are very closed towards alien conspecifics. Because the colonies are monogynous, we suggest that this intolerance may be due to a high intracolonial genetic relatedness. However, monogyny is not systematically associated with a high colonial closure. In *C. cursor*, which is monogynous but monodomous (Cagniant, 1976), very little aggressive behavior is observed when the individuals come from the same site (Nowbahari and Lenoir, 1984). This low level of aggressive behavior can be explained by its mode of colony foundation, which is achieved through fission, resulting in high genetic relatedness between proximate colonies (Lenoir et al., 1988). The high level of colonial closure in *C. iberica* might have originated from the mode of colony foundation. Some field observations suggest that in this species there is an independent mode of colony foundation (X. Cerdá, unpublished data), which, contrary to fission, results in genetic and geographic proximity being opposed. Thus, even neighboring colonies of *C. iberica* may be genetically dissimilar.

The comparative analysis of cuticular washes revealed quantitative differences in hydrocarbons between the tested colonies. Colonies from the same population (B or M), although distinct from each other, had more similar patterns than between populations. This suggests that nests from Barcelona and Murcia

comprise disparate populations. There is also an indication that the disparity between nests within a population may increase with geographic distance. This was evident from the higher distance in the discriminant analysis (based on the hydrocarbon profile) of one nest from Murcia that was located 200 km away from the others. Moreover, there is no geographical barrier between Barcelona and Murcia, and cuticular profiles of ants collected between the two populations show a quantitative gradual divergence of profiles (Dahbi, unpublished data).

We may link the disparities in cuticular hydrocarbons with aggression exhibited by these ants towards alien ants. We base our assumption on the "Gestalt" model. The individuals that were used in recognition tests are nestmates of those analyzed through chromatography, and we assume the presence of a common colonial odor for workers of the same colony (Soroker et al., 1994). The fact that aggression between colonies was at a maximum irrespective of the degree of similarity in hydrocarbons profile suggests that even the small differences exhibited between the colonies of the Barcelona population are above the recognition threshold and suffice to elicit a full response. This hypothesis, however, has to be tested by using pure hydrocarbons at various quantitative combinations.

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EFFECT OF ETHANOL AND α-PINENE ON RESPONSE OF AMBROSIA BEETLE, *Trypodendron lineatum*, TO LINEATIN-BAITED FUNNEL AND DRAINPIPE TRAPS

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Abstract—The response of *T. lineatum* to ethanol and α-pinene, when used with the pheromone lineatin, was tested for two trap types. Funnel traps, which are passive barrier traps, caught significantly more beetles than drainpipe traps, which require an active response by the beetle to enter the trap. However, the response of *T. lineatum* to the semiochemical treatments did not significantly differ for the two trap types. Treatments that included α-pinene with the pheromone, either with or without ethanol, caught significantly more *T. lineatin* than those with the pheromone alone. When ethanol and or α-pinene were added to the pheromone significantly more female beetles were trapped than with pheromone alone. Male–female ratios were significantly lower for both types of traps when ethanol was included in the bait than for lineatin alone or with α-pinene. A higher percentage of male beetles entered the drainpipe traps than was captured with funnel traps.

Key Words—*Trypodendron lineatum*, Scolytidae, ambrosia beetle, lineatin, ethanol, α-pinene, pheromone, semiochemical, Coleoptera, trapping.

INTRODUCTION

The ambrosia beetle *Trypodendron lineatum* (Oliv.) (Coleoptera: Scolytidae) is one of the most significant pests causing damage to commercial wood products

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in the northern hemisphere (McLean, 1985). Although considerable effort has been expended investigating the roles of ethanol and α-pinene as primary attractants (Birch, 1978) and as synergists to the response of *T. lineatum* to its pheromone, lineatin (MacConnell et al., 1977), contrary experimental results continue to confuse the issue, particularly in North America. Understanding the role of ethanol and α-pinene in the chemical ecology of *T. lineatum* is important because commercial mass-trapping programs using semiochemical baits are becoming a common component of ambrosia beetle management programs in the wood products industry.

Moeck (1970, 1971) identified ethanol as a primary attractant for *T. lineatum* in laboratory and field tests but found α-pinene to be repellent in laboratory tests. Bauer and Vité (1975) found both ethanol and α-pinene to be mildly attractive to *T. lineatum* when tested in the field separately, but noted a 10-fold synergistic response when the two chemicals were combined. The individual and synergistic responses to these two chemicals in both western Canada and West Germany were confirmed by Nijholt and Schönherr (1976) and in Sweden by Schroeder and Lindelöw (1989).

There is general agreement that (+)-lineatin is the pheromone of *T. lineatum* in both North America and Europe and that the (−)-enantiomer is inactive, because of similar responses to the (+)-enantiomer and the racemic mixture (Borden et al., 1980; Payne et al., 1983).

In continental Europe, the addition of ethanol and α-pinene to both barrier and tree trunk-simulating (drainpipe) traps baited with lineatin has resulted in increased catches of *T. lineatum* over traps baited with lineatin alone (Vité and Bakke, 1979; Klimetzek et al., 1980; Bakke, 1983; Paiva and Kiesel, 1985). From Canada and England, however, conflicting results were reported. Borden et al. (1982) found in Canada that *T. lineatum* catches in both sticky wire mesh traps and drainpipe traps baited with lineatin were not increased by the addition of α-pinene and decreased with the addition of ethanol. In England, Borden et al. (1982) found that lineatin plus ethanol outperformed the other combinations in fin barrier traps and that the addition of α-pinene inhibited response to lineatin in these traps. In drainpipe traps, lineatin, with either or both α-pinene and ethanol, caught more beetles than lineatin alone, but the increased response to the ternary mixture over lineatin alone was primarily attributed to the effects of ethanol. Drainpipe traps baited with lineatin alone caught fewer females and no more males than those baited only with ethanol and α-pinene in England (Borden et al., 1982).

Further work in Canada by Shore and McLean (1983), using drainpipe traps, indicated a significantly greater catch of *T. lineatum* when both ethanol and α-pinene were added to lineatin than when the pheromone was used alone. Salom and McLean (1988), however, found no significant differences between *T. lineatum* catches with lineatin alone or with any combination of ethanol or

α-pinene in field tests with funnel (barrier type) traps in Canada. They also found no increase in response when ethanol was added to lineatin in funnel traps tested in a wind tunnel. When drainpipe traps were used in a tunnel, however, they found that ethanol stimulated females to enter the traps (Salom and McLean, 1990).

Two possible hypotheses for the disparity in experimental results in these European and North American experiments have been put forward: (1) The beetles are responding differently to the baits due to the different types of traps used in the studies. The glass barrier, fin, funnel, and sticky traps are passive types, i.e., beetles simply hit the side of the trap and drop in or stick to it. Drainpipe traps, however, require an active response by the beetle to crawl into one of the many small entrance holes in order to be trapped. It has been hypothesized that lineatin is sufficient to attract beetles to barrier traps but the addition of the host semiochemicals ethanol and α-pinene is required to induce beetles into the holes of the drainpipe trap (Vité and Bakke, 1979; Salom and McLean, 1990). (2) The beetles' response to ethanol and α-pinene can vary significantly as a result of the release rate of the two semiochemicals. A reduced response to traps baited with α-pinene has been noted in a number of studies (Moeck, 1970; Borden et al., 1982; Bakke, 1983). Bakke (1983) found that release rates of approximately 30 mg/day of α-pinene significantly increased the response of *T. lineatum* to its pheromone, whereas release rates of approximately 100 mg/day reduced catches. He suggested that a high release rate of α-pinene in the English experiments (Borden et al., 1982) might account for the poor response to this semiochemical. Likewise, Salom and McLean (1990) suggested that a high release rate of ethanol (1 g/day) used by Borden et al. (1982) in North America may have produced the repellent effect of this semiochemical in drainpipe traps in their experiment as opposed to the positive results they found using 150 mg/day. Moeck (1970) noted in bioassays that ethanol at high release rates was at first attractive to the beetles, but with continued exposure they became anosmic, probably due to anesthetization of their olfactory sensilla.

In this experiment we tested the hypothesis that the primary attractants ethanol and α-pinene play a more important role in attracting ambrosia beetles into active-type drainpipe traps than into passive-type funnel traps when the semiochemicals are released at comparable rates from the two trap types at rates that are considered to be in the optimal range for commercial trapping purposes (Phero Tech Inc., Delta, British Columbia, Canada).

METHODS AND MATERIALS

A replicated experiment was established at a dryland log sorting area near Duncan, British Columbia. Each of the two replicates consisted of four Lindgren

multiple-funnel traps (Phero Tech Inc.) (Lindgren, 1983) and four Scandinavian drainpipe traps (Borregaard A.S., Sarpsborg, Norway, 1980 model) with the four traps in each trap type baited with one of four semiochemical lure combinations: (1) lineatin, (2) lineatin plus ethanol, (3) lineatin plus α-pinene, and (4) lineatin plus ethanol plus α-pinene.

Lineatin was dispensed from two Hercon laminated dispensers, each releasing at 100 μg/24 hr. (All release rates determined by Phero Tech Inc.) Ethanol was released from a 2.5 × 44-cm PVC pouch at 140 mg/24 hr. The experiment was set up and analyzed in an 8 × 8 Latin square design with time and trap position as the rows and columns (Perry et al., 1980; Shore and McLean, 1983). Traps were placed in the field on April 19, 1989, and beetles were collected on April 28, May 4, May 10, May 15, May 25, June 2, June 12, and June 26. After each beetle collection, the eight traps in each replicate were rearranged until all eight treatment and position combinations had been tested. *T. lineatum* were separated from other insects caught in the traps and counted and sexed in the laboratory. The numbers of beetles caught were transformed to $\log_{10}(x + 1)$, and the percentage of male beetles was transformed to arcsin $\sqrt{x}$ to correct for heteroscedasticity in the data sets before being analyzed by analysis of variance. The analysis was conducted separately for male, female, and total numbers of beetles. Student-Newman-Keul's test was used to test for differences among means. All treatment effects and differences among means were tested at $P \leq 0.05$.

RESULTS

The analysis of variance for the number of female, male, and total beetles captured showed significant effects for replicate (square), semiochemical bait, trap type, trap position, and time (Table 1). There were no significant semiochemical bait × trap type interactions, which indicated that the bait effects were the same for both trap types (Table 1). Traps baited with α-pinene plus lineatin or α-pinene plus ethanol plus lineatin caught significantly more beetles than those baited with lineatin alone. Traps baited with ethanol plus lineatin were intermediate in total beetle response (Table 2). For female *T. lineatum*, treatments containing the pheromone plus ethanol and/or α-pinene captured significantly more beetles than traps baited with lineatin alone. Male beetles responded to the pheromone plus α-pinene or the pheromone plus α-pinene plus ethanol in significantly greater numbers than to the pheromone alone. The addition of ethanol alone to lineatin resulted in a slightly higher, but not statistically significant, response by male *T. lineatum* with the drainpipe trap. (Table 2).

The analysis of variance for the percentage of total beetles captured that were male showed significant effects for semiochemical bait ($F = 9.7$, $dp = 3$,

TABLE 1. *F* VALUES AND PROBABILITIES FOR ANALYSIS OF VARIANCE OF NUMBERS OF *T. lineatum* TRAPPED USING FOUR SEMIOCHEMICAL BAIT TREATMENTS AND TWO TRAP TREATMENTS IN REPLICATED LATIN SQUARE WITH TIME AND TRAP POSITION FOR ROWS AND COLUMNS[a]

		Female		Male		Total	
Source	*df*	*F* value	*P*	*F* value	*P*	*F* value	*P*
Square	1	37.6	<0.001	40.3	<0.001	46.6	<0.001
Bait	3	6.4	<0.001	4.9	<0.01	5.3	<0.01
Trap	1	54.1	<0.001	13.7	<0.001	25.0	<0.001
Bait × trap	3	1.2	0.3274	0.3	0.8366	0.4	<0.001
Position (square)	14	2.9	<0.001	2.9	<0.01	3.2	<0.001
Time (square)	14	19.3	<0.001	22.5	<0.001	24.0	<0.001

[a] Data converted to $\log_{10}(x + 1)$ for analysis.

TABLE 2. TOTAL NUMBER OF *T. lineatum* BEETLES TRAPPED FOR FOUR COMBINATIONS OF SEMIOCHEMICAL BAIT AND PERCENTAGE OF MALE TRAPPED BEETLES, SHOWN SEPARATELY BY TRAP TYPE

Bait[a]	Male (Mean ± SE)[b]	Female (Mean ± SE)	Total (Mean ± SE)	% Male (Mean ± SE)
Funnel traps				
L	151.7 ± 37.5c	82.0 ± 22.6b	233.7 ± 59.7b	65.4 ± 1.9a
L + E	144.8 ± 41.1bc	92.4 ± 25.9a	237.2 ± 66.4ab	59.3 ± 1.9b
L + P	185.9 ± 43.0a	83.2 ± 18.0a	269.1 ± 59.9a	68.6 ± 1.8a
L + E + P	155.2 ± 32.9ab	92.1 ± 21.2a	247.3 ± 53.0a	62.3 ± 1.8b
Drainpipe traps				
L	80.3 ± 27.0c	22.7 + 7.6b	103.0 ± 34.4b	77.4 ± 2.2a
L + E	106.2 ± 35.4bc	55.8 ± 22.6a	162.0 ± 57.7ab	69.1 ± 1.6b
L + P	128.6 ± 33.8a	42.2 ± 12.6a	170.8 ± 44.6a	77.2 ± 3.2a
L + E + P	115.1 ± 34.2ab	53.5 ± 15.3a	168.8 ± 49.0a	67.9 ± 3.4b

[a] L = lineatin, E = ethanol, P = α-pinene.

[b] Means in the same column followed by different letters are significantly different, Student-Newman-Keul's test $P < 0.05$. Number of beetles transformed to $\log_{10}(x + 1)$ and percentage male transformed to arcsin $\sqrt{x}$ for ANOVA.

TABLE 3. NUMBERS OF MALE, FEMALE AND TOTAL *T. lineatum* BEETLES TRAPPED AND PERCENTAGE OF MALE TRAPPED BEETLES FOR FUNNEL TRAPS[a] AND DRAINPIPE TRAPS[b]

Trap type	Male (Mean ± SE)[c]	Female (Mean ± SE)	Total (Mean ± SE)	% Male (Mean ± SE)
Funnel	159.4 ± 19.0a	87.4 ± 10.8a	246.8 ± 29.3a	63.9 ± 1.0a
Drainpipe	107.6 ± 16.1b	43.5 ± 7.7b	151.1 ± 23.3b	72.9 ± 1.4b

[a] Phero Tech Inc., Delta, British Columbia, Canada.
[b] Borregaard A.S., Sarpsborg, Norway.
[c] Means in the same column followed by different letters are significantly different, Student-Newman-Keul's test $P < 0.05$. Numbers of beetles transformed to $\log_{10}(x + 1)$ and percentage male transformed to arcsin $\sqrt{x}$ for ANOVA.

91; $P < 0.001$), trap type ($F = 40.6$, $df = 1, 91$; $P < 0.0001$) and time ($F = 3.3$, $df = 1, 91$; $P < 0.001$). There was no significant bait by trap type interaction ($F = 1.0$, $df = 3, 91$; $P > 0.40$), which indicated that the sexes responded to the different semiochemical baits similarly for both trap types. The addition of ethanol to traps baited with lineatin alone, or lineatin plus α-pinene, resulted in a significant decrease in the percentage of total beetles captured that were male (Table 2).

The total number of beetles, both males and females, captured in the Lindgren funnel traps was significantly greater than the number caught in the drainpipe traps (Table 2). A higher percentage of male beetles was captured in the drainpipe traps than in the funnel traps (Table 3).

DISCUSSION

The finding that funnel traps caught more *T. lineatum* than drainpipe traps in North America confirms previous reports by Lindgren et al. (1983) and McLean et al. (1987). These workers were unable to show statistically significant differences for both sexes, however. Our results show statistically significant differences for both sexes separately as well as for the total catch. The higher percentage of males captured in drainpipe than in barrier traps has been previously reported from Europe (Klimetzek et al., 1980). This result can probably be explained because the female is the first-attacking sex and the male is conditioned to enter the galleries initiated by the females.

The hypothesis that *T. lineatum* response to ethanol and α-pinene when combined with lineatin was related to active or passive trap type was rejected. Beetles responded in a similar manner to the bait combinations in both trap types. The results also confirm the finding of Shore and McLean (1983) that

drainpipe traps baited with the tertiary combination caught more beetles than those baited with lineatin alone. This finding is now extended to funnel traps in North America, which is in disagreement with results reported by Salom and McLean (1988).

The addition of ethanol resulted in a higher percentage of female beetles responding. This has been reported by others (Borden et al., 1982; Bakke, 1983) and probably reflects the chemical ecology of the insect. The female is the pioneer sex in *T. lineatum* and responds to logs in which fermentation has started (Graham, 1968). Thus host odors may be more important to the female and the pheromone more important to the male (Salom and McLean, 1990). In our experiment, however, ethanol increased female response but α-pinene did not. α-Pinene was the more important of the two kairomones in increasing total *T. lineatum* response, but this increase was more in the number of males than females. As *T. lineatum* brood studies have shown a sex ratio of 1 : 1 (Shore et al., 1987), none of the bait and trap combinations attracts sufficient females to maximize mass-trapping efficiency.

In Europe, ethanol and α-pinene have consistently attracted *T. lineatum* either on their own or synergistically with each other and/or the pheromone lineatin in both passive and active trap types (Bauer and Vité, 1975; Nijholt and Schönherr, 1976; Vité and Bakke, 1979; Klimetzek et al., 1980, 1986; Borden et al., 1982; Bakke, 1983; Paiva and Kiesel, 1985; Ramisch and Bombosch, 1986; Schroeder, 1988; Kvamme, 1986; Schroeder and Lindelöw, 1989).

In North America, however, there have been a number of conflicting experimental results involving *T. lineatum* response to ethanol and α-pinene. There are several possible explanations for the differences in experimental results within North America and between North America and Europe. Some of these have been tested and rejected. For example, there is no evidence to suggest different responses to enantiomers of α-pinene or lineatin by North American and European *T. lineatum* (Payne et al., 1983; King et al., 1983; Andryszak et al., 1987). The results reported here suggest that *T. lineatum* responds similarly to semiochemicals whether they are in active or passive trap types, although the sex ratios responding to the two trap types are somewhat different. In North America, experimental design and release rates are two possible factors that may have contributed to conflicting results.

Borden et al. (1982) used a randomized block design and Shore and McLean (1983), Salom and McLean (1988), and the experiment reported here used a Latin square design with time and trap position as the rows and columns. This latter experimental design has the advantage of separating treatment effects from the variation attributed to differences in trap location and time in the analysis of variance, thus making the test more sensitive to treatment differences (Perry et al., 1980). This does not provide an explanation, however, for the difference in results between the findings of Salom and McLean (1988), in which the

addition of ethanol and α-pinene did not enhance trap catches of *T. lineatum*, and the experimental results reported here.

The release rates of lineatin, ethanol, and α-pinene have differed in the North American experiments. Borden et al. (1982) used 1 g/day of ethanol in some experiments, which is considerably higher than the release rates used in other experiments and may explain the repellent response of *T. lineatum* to this bait in those experiments. In other experiments, however, they tested ethanol and α-pinene at lower rates that were later found to be within the optimal range (Bakke, 1983; Lindgren et al., 1983; Klimetzek et al., 1986), and although there was a trend to higher catches with the lower rates, there was still no improvement over the pheromone alone. Further work on optimizing release rates is needed. In Europe, a variety of release rates, experimental designs, and trap types have been used, and the response of *T. lineatum* to ethanol and α-pinene has appeared to be much more consistent.

In Europe, interspecific competition by *T. lineatum* with the hardwood-inhabiting species *T. domesticum* (L.) and *T. signatum* (F.) may have led to stronger host kairomone responses than in North America. *T. lineatum* commonly attacks hardwood species in North America (Wood, 1982; Lindgren, 1986), whereas in Europe this is considered rare (Å. Lindelöw,[2] Forest entomologist, Department of Entomology, Swedish University of Agricultural Sciences, Uppsala, Sweden, personal communication). All three species respond to and produce (+)-lineatin (Klimetzek et al., 1981; Schurig et al., 1982), so there likely is a need for additional compounds to maintain reproductive isolation. Tømmerås (1989) showed that *T. lineatum* has receptors sensitive to volatiles from the nonhost birch. Birch is a host of *T. domesticum*, which was found to be inhibited by α-pinene (Nijholt and Schönherr, 1976).

Our study does not explain discrepancies among North American findings on the role of host kairomones as synergists to lineatin for *T. lineatum*, but it supports the use of host volatiles in commercial trapping programs. It appears that host kairomones are not as important to North American *T. lineatum* as to their European conspecifics. Nevertheless, under certain circumstances, host volatiles play a significant role in the chemical ecology of this species in North America.

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ROLE OF PREY ODOR IN FOOD RECOGNITION BY ROCK CRABS, *Cancer irroratus* SAY

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Abstract—Rock crabs, *Cancer irroratus*, respond to food odors in low concentration as measured by changes in antennular flicking rate. The responses of rock crabs to prey odor were tested in the presence and absence of visual cues to determine the role of chemical cues in prey recognition. Crabs were attracted to the source of mussel odor introduced into one arm of a Y maze. Natural and artificial prey shells and resin boxes were presented to crabs with and without the presence of mussel extract. The crabs were able to see, handle, and manipulate these objects. Crabs opened and consumed contents that emitted chemosensory cues and ignored identical objects that did not. Rock crabs were attracted to food odors and are capable of utilizing chemical cues to detect, locate, and identify food items.

Key Words—Crustacea, crabs, marine animals, chemoreception, odor, vision, foraging, feeding, *Cancer irroratus*.

INTRODUCTION

Efficient foraging requires that animals expend minimum amounts of energy searching for, handling, or subduing prey and investigating or attempting to feed upon nonfood objects. An efficient or optimally foraging crab should be able to detect cues from distant prey, follow these cues to their source, and then be able to differentiate edible shelled prey from empty shells and other inedible environmental objects such as small stones. Attempting to open extraneous environmental objects increases the possibility of claw damage, which would impair future foraging (Juanes and Hartwick, 1990).

In the mid-Atlantic region, rock crabs, *Cancer irroratus*, migrate annually from depths of up to 700 m to shallower inshore areas (depths of 1–20 m) in

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October–November and remain until March–April (Shotton, 1973; Haefner and van Engel, 1975). While in deeper offshore waters, or in turbid, shallow waters, there is insufficient light for food detection by visual mechanisms. Because rock crabs are crepuscular and nocturnal foragers (Rebach, 1985, 1987), vision may not be very useful even in clear, shallow environments.

Crustacea utilize chemoreception in both the finding of food and the initiation of feeding. They detect food by distance chemoreception using their antennules and by contact chemoreception using chemosensory hairs on antennules, carapace, mouthparts and legs (Rittschoff, 1992). Chemical cues released from prey (body odors) provide appropriate stimuli for investigative and attractive phases of feeding, while prey extracts provide natural stimuli for studying ingestive phases of feeding (Zimmer-Faust, 1993). Recent reviews of chemoreception and feeding behavior in crustaceans can be found in Ache (1982, 1988), Carr and Derby (1986), Derby and Atema (1988), and Laverack (1988). Rittschoff (1992) reviews how social, environmental, and physiological contexts determine the result of chemosensory input in the daily life of crabs.

Vision is commonly used in aggressive displays by *Callinectes sapidus* (Jachowski, 1974) and *Carcinus* (Lee and Seed, 1992), and in mating by *Callinectes* (Jachowski, 1974; Milliken and Williams, 1984) and *Cardiosoma* (Ameyaw-Akumfi, 1987). In the hermit crab, *Clibanarius vittatus*, chemical cues from gastropod flesh initiate visually directed shell-seeking behavior in crabs with poorly fitting shells. *Clibanarius* is capable of visually discriminating shapes related to the potential habitability of gastropod shells (Diaz et al., 1994). Langdon and Herrnkind (1985) have demonstrated that the fiddler crab, *Uca pugilator*, is also capable of shape discrimination. Generally, *Callinectes* is a visually oriented species, found in shallow waters where light penetration is good. From underwater, they are capable of capturing the marsh periwinkle, *Littorina*, when the snail is out of water on *Spartina* stems (Hamilton, 1976).

My current research attempts to determine the relative roles of visual and chemosensory modalities in: (1) prey detection, (2) prey location, and (3) prey identification and to consider decision rules used by foragers in responding to food attractants.

METHODS AND MATERIALS

Subjects and Maintenance Conditions. Male rock crabs, *Cancer irroratus*, in intermolt stage and with intact claws, were used for all experiments. Each animal was used only once. Crabs were maintained in 30‰ artificial seawater (Dayno Manufacturing, Inc.) at ambient seasonal temperatures and fed Crustacean Ration (Rebach, 1981) daily, and ribbed mussels (*Geukensia demissa*) once per week. Test crabs were starved for 24 hr to standardize hunger levels prior to testing.

Attraction to Odors. A Y maze 91.4 cm long constructed of 1.2 cm thick Plexiglas was used. Water entered through 6-mm-diameter ports 2 cm from the bottom of the tank, and exited from a 12-mm-diameter opening 12.5 cm from the bottom. A Masterflex dual-head peristaltic pump delivered water at 330 ml/min (165 ml/side) (Figure 1). In control tests, crabs indicated no side preference. Dye studies showed that little mixing occurred at the base of the Y within the 10-min testing time.

Unfiltered seawater entered through one port, and seawater that had passed over three 4.0-cm, freshly cracked ribbed mussels entered through the other. The maze arm dispensing the seawater exposed to mussels was varied randomly. The test tank was drained, washed, and dried between each test.

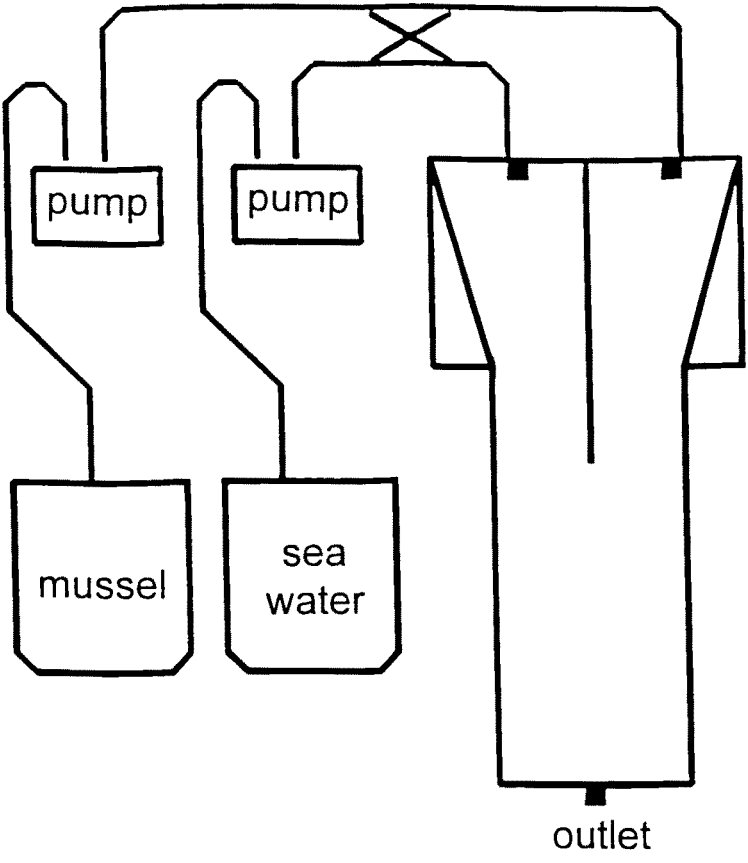

FIG. 1. Setup of Y maze and water delivery system. Samples could be delivered to either arm of the Y maze.

A crab was placed at the base of the Y maze under a weighted, perforated plastic cup (15 cm diameter, 7 cm high) for 10 min before the experiment began. The cup remained in place for 1 min after water flow commenced, then was lifted gently with a remotely operated string.

A video camera, with a wide-angle lens, was positioned above the tank and connected to a monitor and video recorder several feet away. Each crab was observed for 10 min through the monitor, and position and elapsed time were recorded on a schematic diagram of the tank whenever a position change occurred. Choice of a side was determined when a crab spent more than 75% (>7.5 min) in one arm of the chamber beyond the divider. A videorecording of the behavior was made for later reviewing, if necessary.

Food Recognition. Testing was conducted in a 38-liter glass aquarium (50.8 × 25.4 cm), filled $\frac{1}{2}$ full (15 cm) with seawater (30‰) at 12°C. The aquarium had a clear Plexiglas divider, which could be inserted or removed, 13.5 cm from one end.

Crabs were presented with a choice between an odorless prey item (a clean, empty, 4.5-cm-long mussel, boiled for 10 min, and valves reglued) and an item that released prey odor (a sponge soaked in *Geukensia* extract and placed in a small, 3.3 × 3.3 × 1.5 cm opaque, resin box). The opaque box was molded of ProCast 10 (Rudolf Bros. & Co., 960 Walnut St., P.O. Box 175, Canal Winchester, Ohio 43117), a bone white urethane casting resin. Mussel extract was prepared by macerating a freshly caught mussel immediately before the test. From crab eye-level, the mussel and the box offered equal presentation areas. The box had five 3-mm holes drilled in the lid, and contained a sponge soaked in mussel extract. The box and sponge weighed 6.5 g, which approximates the weight of a 4.5-cm mussel.

Experimental crabs were starved and acclimated to the testing tank, with divider in place, for 24 hr before testing. Ten minutes before the experiment began, a box with mussel-extract-soaked sponge and a clean mussel shell were placed at opposite sides of the tank behind the divider. The divider was then raised and the crab's response was monitored. Again, the crab was observed through a video monitor, which offered a dorsal view, and through the side of the tank. When the crab picked up an object and crushed or broke it open, it was considered to have made a choice. If the crab failed to make a choice after 1 hr, the experiment was terminated.

Crabs were subsequently given the choice between boxes with chemical cues (sponge) and empty mussel shells with chemical cues (sponge) to determine if, when odor was held constant, crabs would prefer objects that had the appearance of prey.

Because it is possible that the boiled, dried, mussel shells still provided chemical information from shell proteins, faux mussel shells were cast from the same resin used to make the boxes. Real mussel shells were tested against resin

mussel shells, without using odor-impregnated sponges. Odor was held constant, being absent in both choices. Prior to using the resin shells, several crabs were offered live mussels and faux mussels containing sponges and mussel extract. Each type was consumed in equal amounts. All test series were scheduled to be conducted until 15 individuals had made a choice. Preferences were determined by using a two-tailed binomial test (Siegel and Castellan, 1988).

RESULTS

Control tests ($N = 13$) with the Y Maze indicated that a side preference did not exist. However, when given the choice between seawater that had passed over mussels and control seawater of the same salinity, 10 crabs entered the arm of the maze that dispensed seawater + mussel odor and two entered the seawater arm ($P = 0.038$, $N = 12$). Crabs were frequently observed attempting to climb onto the water dispenser disbursing prey odor and exhibited increased activity upon reaching the test water input port.

When presented with the choice of a box containing a sponge and mussel extract, and dried, empty mussel shells, 12 crabs chose the box, three chose the mussel shell, and three others made no choice (Figure 2A, $P = 0.036$, $N = 15$). A choice was scored when a crab either opened the box or opened the mussel. Most of the crabs crushed the box to get to the mussel-soaked sponge but several were capable of using their chelae to open the box. Although boxes and shells were weighted to approximate the natural weights of mussels of that size class, sponge cubes were less dense than seawater and often floated to the surface of the test tank. At least half the crabs grasped the sponge with their chelae and proceeded to rip it into small pieces, many of which were consumed. The three animals that made no choice remained motionless in the tank. In every case in which a crab handled prey, it made a choice. Crabs not making choices were not considered in the data analysis.

Crabs given a choice between boxes and mussel shells, each containing sponges soaked in mussel extract, and each releasing chemical cues, chose randomly. Seven crabs chose the box with sponge, and eight animals chose the mussel shell with sponge (Figure 2B, $P = 1.0$, $N = 15$). One animal made no choice during the testing period.

For the choice tests between real and faux mussel shells (both without chemical cues), I was unable to test enough crabs to achieve the designated cutoff of 15 animals making a decision before the season ended and crabs became unavailable. Seven crabs chose the real mussel, three chose the faux mussel shell, and 25 made no choice (Figure 2C, $P = 0.344$, $N = 10$). After 25 animals failed to make a choice, this series of tests was abandoned. This large a percentage of animals not making a choice was not observed in any previous

A.

BOX	MUSSEL SHELL	NO CHOICE
ODOR	NO ODOR	
12	3	3
p = 0.036 *		

B.

BOX	MUSSEL SHELL	NO CHOICE
ODOR	ODOR	
7	8	1
N.S.		

C.

MUSSEL SHELL	FAUX SHELL	NO CHOICE
NO ODOR	NO ODOR	
7	3	25
N.S.		

FIG. 2. Food recognition choice tests. Probabilities calculated by two-tailed binomial test. NS = nonsignificant, $*P < 0.05$. (A) Choices made between resin box containing sponge soaked in mussel extract and a clean, boiled mussel shell. (B) Choices made between resin box containing sponge soaked in mussel extract and a clean, boiled mussel shell containing sponge soaked in mussel extract. (C) Choices made between a clean, boiled mussel shell and a faux resin mussel shell of the same size.

series. The number of crabs making choices when prey odor was present was compared to the number making choices when odor was not present by the Fisher's exact test (Sokal and Rohlf, 1981) and differed significantly ($P < 0.0001$). I then introduced cracked, but unopened, live mussels to crabs that were inactive during the 1-hr test period. These crabs initiated activity, picked up the cracked mussels, opened, and consumed them.

DISCUSSION

Previous work has indicated that rock crabs detect the presence of mussel extract at concentrations as low as 10^{-10} g/liter, measured by increases in antennal flicking rate at increasing concentrations of extract. Concentrations greater than 10^{-7} g/liter induced feeding behavior (Rebach et al., 1990), indicating the ability of crabs to detect and differentiate odors of varying strength from a distance. Increased concentrations of food odors may reflect increased concentrations or value of foods and influence aspects of foraging behavior (Zimmer-Faust, 1989). Chemical cues are important attractants and may be correlated with probable yield and vulnerability of prey. Using models, Kaiser et al. (1993) found that the more concentrated the odor, the greater the preference, the greater the number eaten, and the more time spent handling the model.

The Y maze experiments indicate that rock crabs orient toward and are attracted to prey odor. Prey body odors are ecologically relevant stimuli in assaying exploratory search behavior by foragers (Zimmer-Faust, 1989).

Although crabs may use chemical cues to detect and approach prey, they must still determine which environmental objects are edible and which are not upon arriving at the source of the odor. By choosing boxes and shells containing sponges soaked in mussel extract with equal frequency, crabs apparently did not utilize visual cues in the short-range prey discrimination tests. If visual cues were used in conjunction with odor, mussel shells would have been chosen disproportionately. When mussel extract was not present in mussel shells, crabs chose boxes that released food odors over mussel shells that did not. *Carcinus* has been observed to bite off pieces of plaster models containing mussel homogenate and persisted longer with scented than with odorless models (Kaiser et al., 1993). Prey extracts provide the most natural stimuli for assaying the ingestive phase of feeding, since substances released by the extract are likely to be similar to those escaping as predators consume prey (Zimmer-Faust, 1989). These compounds influence feeding behavior differently in different species. ATP is commonly present in prey tissue extract and rapidly degrades to AMP, which acts as a repellent in the spiny lobster, *Panulirus interruptus* (Zimmer-Faust, 1993), and as a feeding behavior stimulant in *Palaemonetes pugio*, an opportunistic scavenger (Carr and Derby, 1986). *Cancer irroratus* is a predator

and a scavenger (Bigford, 1979), whose diet typically includes hard-bodied prey such as crustaceans, bivalves and gastropods (Hudon and Lamarche, 1989; Stehlik, 1993), and should be attracted by the release of tissue extracts (Rittschoff and Sutherland, 1986).

Tests with empty shells may not be proper controls since mussel shells contain proteins, such as conchiolin, secreted by the mantle (Wilbur and Saleuddin, 1983), and chemical cues might be available even in shells that had been scraped and oven dried. Shells cast of resin allowed removal of all naturally occurring proteins from the test situation. The crabs seldom chose between real shells and faux shells without implanted odors. They often failed to move and appeared unwilling to handle and open environmental objects without appropriate chemical cues to stimulate feeding behavior. The comparison of crabs making choices to those not choosing indicated that, in these experiments, the presence of odor was necessary for making a choice and that visual cues were not sufficient to stimulate investigation of prey. Visual cues did not promote or stimulate feeding activity. The lack of movement was apparently not a matter of lack of hunger because crabs exhibited normal feeding responses when cracked mussels were presented. It appears that the lack of choice was prompted by the lack of chemical cues.

Rock crabs can detect the presence of mussel extract in very low concentrations. The Y maze tests indicate that they are attracted to the source of this odor without the presence of any visual information. When presented with choices in which some objects emitted prey odor, crabs chose these objects whether they were shells of real prey, artificial shells resembling actual prey, or boxes that resemble prey neither in color, shape, nor texture. Likewise, real and artificial objects that did not emit chemosensory information were generally ignored. In fact, crabs often stood on empty mussel shells to facilitate reaching resin boxes. Stehlik (1993), however, found objects of human origin such as fibers, hairs, wood, plastic beads, and plastic sponges in the guts of *C. irroratus* and *C. borealis*. Patches of prey often contain inedible items such as stones and empty shells, and foragers will encounter prey and debris simultaneously and might be fooled by pervasive odors. While feeding, the material being consumed is usually beneath them, making it rather difficult to employ visual cues. Handling times decrease as learning occurs, and crabs can probably learn to avoid inedible objects from experience (Kaiser et al., 1993). When given the choice between real and artificial "covers" for odor sources, choices were made, but no preference was exhibited. When given the same choice without an odor source, no choices were made.

This study indicates that foraging rock crabs make several decisions based on questions such as: (1) is food present? (2) where is it? (3) should it be approached? and (4) is it edible? It appears that chemical cues are necessary

and sufficient for the detection, location, and identification of food items by rock crabs and that visual cues are ignored.

It is interesting to note that in other work in our laboratory, crabs chose larger prey over smaller prey when neither had any associated chemical cues. Visual cues may be utilized to distinguish prey from other environmental objects in the immediate vicinity of the prey, although the foreign objects found in crab guts by Stehlik (1993) might argue otherwise.

Vision may play a role in initiating an appropriate response to a physiological input since social and environmental contexts can influence the responses to chemosensory input. Vision may be used for discrimination when similar odors are produced by living, or recently deceased, predators. The odor of dead conspecifics and living competitors or predators may indicate danger and influence behavior. Responses to odors can also be influenced by molt stage, reproductive stage, and hunger level (Zimmer-Faust, 1989).

My experiments were unable to elucidate the role of visual cues in prey selection. Rock crabs appear to be essentially nonvisual predators. Chemical information directs these crabs to their prey and may provide information about prey quality or quantity. Prey quality may be evaluated by tactile or contact chemoreception, resulting in predatory efficiency by reducing energy expenditure on unprofitable prey items (Kaiser et al., 1993). The data indicate that rock crabs are sensitive to and attracted by food odors and locate, recognize, and identify prey by chemosensory, rather than visual, mechanisms. This may be an adaptation to nocturnal activity, the murkiness of the inshore water, and the lack of light penetration in their offshore habitat.

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STRUCTURES AND ALLELOPATHIC EFFECTS OF *Nuphar* ALKALOIDS: NUPHAROLUTINE AND 6,6′-DIHYDROXYTHIOBINUPHARIDINE

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Abstract—Two of the major *Nuphar* alkaloids, nupharolutine and 6,6′-dihydroxythiobinupharidine, were isolated from the aquatic perennial herb *Nuphar lutea* (L.) Sibth. & Sm., yellow water lily. In a lettuce seedling bioassay of the two pure compounds, the former was inactive and the latter was highly inhibitory of radicle elongation at concentrations greater than 2 ppm. Structures and stereochemistry of the two compounds were confirmed by DEPT, ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY, and ^{1}H-^{1}H NOESY experiments.

Key Words—Allelopathy, aquatic plant, bioassay, *Nuphar lutea*, alkaloids, NMR, nupharolutine, 6,6′-dihydroxythiobinupharidine, Nymphaeaceae.

INTRODUCTION

Earlier work in our laboratory surveyed for potential allelopathic activity some 25 aquatic and wetland plants native to the southeastern United States (Elakovich and Wooten, 1989, 1991; Wooten and Elakovich, 1991). An aqueous extract of the aquatic perennial herb *Nuphar lutea* (L.) Sibth. & Sm., family Nymphaeceae, yellow water lily, exhibited the highest allelopathic potential according to lettuce seedling and *Lemna minor* growth bioassays (Elakovich and Wooten, 1991). An extract of yellow water lily was almost 10 times as inhibitory as the second most active aqueous plant extract, that of *Nymphaea odorata* Ait. *Nuphar lutea* is comprised of nine often poorly differentiated subspecies occurring in rivers, estuaries, bayous, sloughs, marshes, swamps, ditches, lakes, and

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ponds of North America and Europe. Where ranges of subspecies overlap, many specimens are intermediate between the overlapping subspecies (Godfrey and Wooten, 1981). Populations of *N. lutea* are often so dense that the water surface is not visible; such populations apparently exclude other hydrophyte growth.

Previous work established that both the leaf and the root and rhizome aqueous extracts of *Nuphar lutea* inhibited *Lemna minor* frond growth and lettuce seedling radicle elongation (Elakovich and Wooten, 1991). Extracts were prepared by blending equal weights of fresh plant material and deionized water and filtering. Inhibition by these *N. lutea* aqueous extracts was found at concentrations of 2.5 parts per thousand (ppt) or greater.

Nuphar lutea produces alkaloids having a regular sesquiterpenic skeleton incorporated into 3-furyl-substituted piperidines or quinolizidines as well as C_{30} alkaloids that contain a sulfur atom along with two 3-furyl substituents (Wrobel and Ruszkowsha, 1979; LaLonde and Wong, 1977; Iwanow et al., 1986). Cullen et al. (1973) examined the antifungal activity of the rhizomes whose medicinal utility had been reported 20 years earlier (Tatarov, 1945). There are no reports on the allelopathic potential of *Nuphar* alkaloids.

Continued discovery of agrochemically active compounds from natural sources supports the notion that new herbicides, insecticides, and antimicrobials may be developed from templates discovered among natural products (Cardellina, 1988). McClure (1970) reiterated the postulation that "Aquatic angiosperms are considered to be descendants of terrestrial plants which have reverted to the aquatic habits of their remote ancestors." He suggested that the initial change from an aquatic to an emergent habitat appears to involve a shift from an essentially anaerobic to an aerobic type of metabolism and would surely be expected to influence the qualitative and quantitative production of secondary constituents. Thus, because aquatic plants occupy different ecological niches than terrestrial plants, they may also produce allelochemicals with different target/nontarget toxicity profiles than terrestrial plants. Some of these biologically active natural products may be developed into commercial herbicides that may be more environmentally acceptable than are present herbicides (Duke, 1986). Data on the plant-growth-inhibitory activity of some 100 species of aquatic and wetland plants have been reported (Elakovich and Wooten, 1995), but there are only a handful of reports on the structure of specific bioactive compounds from these plants.

This paper describes the isolation and full NMR characterization of two of the known alkaloids from *N. lutea* and an examination of the allelopathic potential of these compounds as measured by lettuce seedling bioassay.

METHODS AND MATERIALS

General Experimental Procedures. NMR solution spectra were obtained on a Bruker AC-300 spectrometer using standard pulse sequences and Bruker

software for 2D experiments. IR spectra were obtained in CH_2Cl_2 solution on a Mattson Polaris spectrometer. MS (electron impact) were obtained on a Hewlett Packard 5985 GC/MS system. TLC was performed on 20 cm × 0.25 mm commercial plates with UV detection.

Plant Material. Nuphar lutea sb. *macrophyllum* whole plants were collected from a quiescent pond in Tylertown, Mississippi, during October and November 1991. Plants were washed free of debris, drained of excess water, and divided into two portions: (1) leaves, including blades and petioles; and (2) rhizomes and roots. Plants were dried in a plant dryer, ground to a coarse powder, and stored at room temperature. A voucher specimen was deposited in the University of Southern Mississippi Herbarium, Department of Biological Sciences.

Extraction and Isolation. Two batches of powdered roots and rhizomes of *N. lutea*, 1.7 and 3.0 kg, were extracted according to a method modified from that of Cullen et al. (1973) to yield 17.7 and 26 g of crude alkaloid mixture, respectively. A 5-g portion of the alkaloid mixture was chromatographed over activity III alumina and eluted with hexane (200 ml); hexane-CH_2Cl_2 mixtures as follows: 99:1 (260 ml), 95:5 (525 ml), 90:10 (450 ml), 8:2 (130 ml), 1:1 (470 ml), 1:3 (300 ml); CH_2Cl_2 (1000 ml); and CH_2Cl_2–MeOH 99:1 (200 ml). The CH_2Cl_2–MeOH fraction was rechromatographed over activity II alumina and eluted with CH_2Cl_2 (1000 ml) and CH_2Cl_2–0.4% MeOH (400 ml). The CH_2Cl_2–MeOH fraction was rechromatographed over activity III alumina and eluted with hexane (400 ml), hexane–ether 95:5 (200 ml) and 85:15 (200 ml), and ether (200 ml). The fraction from 85:15 hexane–ether was rechromatographed over activity III alumina and eluted with hexane–ether 95:5 (200 ml) and 90:10 (200 ml). The fraction from 90:10 hexane–ether yielded 28 mg of nupharolutine.

A 15-g portion of the crude alkaloid mixture was chromatographed over activity III alumina and eluted with 1000-ml portions of hexane–triethylamine, 99:1, fractions 1–4; hexane-CH_2Cl_2–triethylamine, 94:5:1, fractions 5–9; 64:35:1, fractions 10–14; 39:60:1, fractions 15–19; 9:90:1, fractions 20–24; CH_2Cl_2–triethylamine, 99:1, fractions 25–29; and CH_2Cl_2–MeOH–triethylamine, 98:1:1, fractions 30–34. Fraction 22 was extracted twice with 100-ml portions of 5% NaOH. The remaining organic phase was concentrated to yield 180 mg of colorless crystals of 6,6′-dihydroxythiobinupharidine.

NMR assignments were made based on DEPT, ^1H–^1H COSY, and ^1H–^{13}C COSY experiments. Stereochemical assignments for nupharolutine were aided by ^1H–^1H NOESY experiments. DEPT spectra allow the identification of carbon signals as methyl groups, methylene groups, methine groups, or quaternary carbons. ^1H–^{13}C COSY spectra allow the correlation of the individual carbon signals with the proton signals of the hydrogen attached to those carbons; ^1H–^1H COSY spectra allow the correlation of hydrogen signals with the other hydrogen signals to which they are coupled. NOESY spectra show signal

enhancement for groups that are close, and thus aid in stereochemical assignments.

Nupharolutine. Mp 102.5–103.5°C. MS *m/e* (% relative intensity) 249 (M^+, 40), 234 (5), 220 (10), 206 (10), 178 (30), 164 (10), 148 (12), 136 (50), 114 (80), 107 (30), 94 (100), 81 (35), 70 (28), 55 (40). These data are consistent with published values (LaLonde et al., 1972; Wrobel et al., 1972a). NMR assignments are given in the Results section.

6,6'-Dihydroxythiobinupharidine. Mp 136.0–139.0°C. MS *m/e* (% relative intensity) 508 (4), 446 (21), 371 (2.4), 337 (2.4), 309 (5.7), 282 (3.3), 268 (3.3), 248 (5.2), 230 (100), 216 (14.2), 188 (3.3), 176 (6.1), 160 (3.8), 148 (5.7), 132 (5.7), 107 (17.0), 94 (86), 81 (25.5). These data are consistent with published values (LaLonde et al., 1970). NMR assignments are given in the Results section.

Bioassays. Lettuce seedling growth bioassays of the crude alkaloid mixture, the two isolated alkaloids, of the known plant growth inhibitor *dl*-2-aminoadipic acid, and of the ethanol solvent were carried out according to previously published procedures (Elakovich and Wooten, 1991) using *Lactuca sativa* Black Seeded Simpson. For the assay of *dl*-2-aminoadipic acid, 10 pregerminated seeds per test plate were used; all other assays used 15 pregerminated seeds per plate. Ethanol added for sample solubilization did not exceed 0.1% of the total 15 ml test volume. Seedlings were allowed to grow at room temperature in the dark for 60–72 hr before either hypocotyls, radicles, or both were measured to the nearest 0.1 cm. Assay results were subjected to analyses of variance and Duncan's multiple-range test.

RESULTS AND DISCUSSION

Nupharolutine, $C_{15}H_{23}NO_2$ **(1),** was isolated from *N. lutea* as glasslike crystals. The ^{13}C NMR and DEPT spectra indicate that the alkaloid has 15 carbons with six methine (δ 143, 140, 110, 69, 60, and 37) five methylene (δ 65, 40, 36, 34, and 29), two methyl (δ 26 and 19), and two quarternary (δ 130 and 68) carbons. The MS indicates a parent ion peak at *m/e* 249 corresponding to $C_{15}H_{23}NO_2$ *Nuphar* alkaloids. The IR contains bands characteristic of hydroxyl absorption (3591 cm^{-1}), the *trans*-quinolizidine ring system (2952 cm^{-1}) (Bohlmann, 1958), and furyl bands (1501–875 cm^{-1}). The ^1H–^1H COSY spin correlations demonstrate that the product could be either nupharolutine **(1)** (Figure 1), or its epimer, 7-epinupharolutine. The IR spectrum of 7-epinupharolutine contains a concentration-independent intramolecular hydrogen-bonding absorption at 3520 cm^{-1}, while **1** displays a hydrogen-bonding absorption at 3520–3300 cm^{-1}, which disappears completely at low concentrations, whereas the band of the epimer persists (LaLonde et al., 1972). The IR spectrum of a dilute (CH_2Cl_2) sample of our product contains no hydrogen-bonding bands, and the

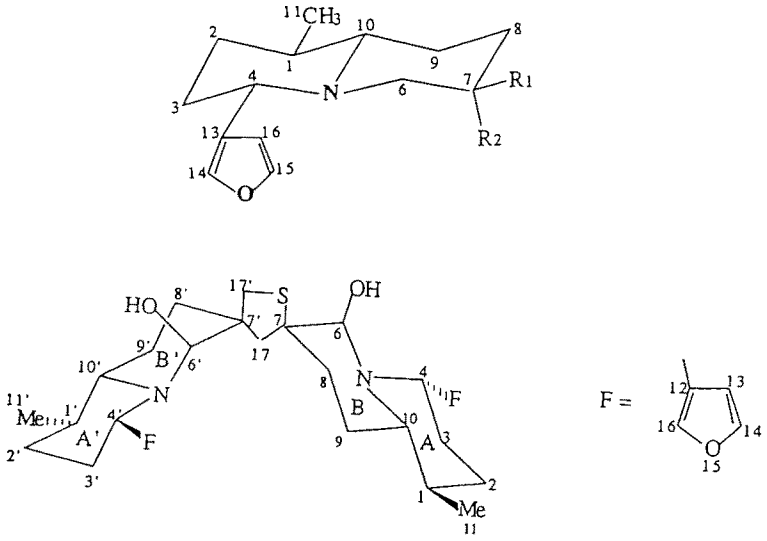

FIG. 1. The structures of nupharolutine (top) and 6,6′-dihydroxythiobinupharidine (bottom). In nupharolutine (top) R_1 = OH and R_2 = CH_3 (C-12); when R_1 = CH_3 (C-12) and R_2 = OH, the compound is 7-epinupharolutine (not isolated or assayed).

MS is consistent with published data for nupharolutine (LaLonde et al., 1972; Wrobel et al., 1972a).

The furyl group chemical shifts are assigned as δ130 (C-13), δ143 (C-14), δ140 (C-15), δ110 (C-16), and δ7.5 (H-14), δ7.4 (H-15), and δ6.4 (H-16) from the ^{1}H–^{1}H and the ^{1}H–^{13}C COSY spectra. The doublet for methyl protons at δ0.9 correlates with the methyl carbon at δ19 (CH_3-11); the methyl carbon at δ26 correlates with the protons at δ1.2 (CH_3-12). Tracing the spin system through the ^{1}H–^{1}H and ^{1}H–^{13}C COSY spectra reveals the proton assignments δ1.4 (H-1), δ1.95 (H-2), δ1.65 (H-3, H-8 and H-10), δ3.0 (H-4), δ1.15 (H-9), and the correlated carbon assignments δ29 (C-2), δ40 (C-3), δ60 (C-4), δ36 (C-8), δ34 (C-9), δ69 (C-10). The remaining three carbons are assigned δ26 (C-12), δ65 (C-6), and δ68 (C-7) from DEPT spectra. The C-6 geminal protons are assigned δ2.70 (H_{eq}-6) and δ1.65 (H_{ax}-6); their COSY cross peak is prominent. The hydroxyl proton occurs at δ3.40.

Stereochemical studies of this compound were focused on C-1, C-4, C-7, C-10, and the nitrogen atom. The strong IR bands at 2952 cm^{-1} support the *trans*-quinolizidine system that defines the stereochemistry of C-10 and the nitrogen atom (Bohlmann, 1958), and its large size requires that the furyl group be equatorial (LaLonde and Wong, 1977). The presence of an H-10 H-11 NOESY cross peak and the absence of CH_3-11-furyl cross peaks demonstrated that the

CH_3-11 methyl group is equatorial. The NOESY cross peak of the C-7 methyl and the furyl H-16 proton suggests that the methyl is axial and confirms that the furyl group is equatorial. Thus, we conclude that compound **1** is nupharolutine.

6,6′-Dihydroxythiobinupharidine, $C_{30}H_{42}N_2O_4S$ **(2)** (Figure 1), was isolated as glasslike crystals. The IR spectrum resembles that of **1.** The ^{13}C NMR and DEPT spectra support a C_{30} alkaloid structure with 2 methyl (δ19.1, 19.2), 10 methylene (δ44.2, 39.6, 35.0, 34.8, 33.4, 33.3, 32.0, 30.3, 29.1, 28.5), 14 methine (δ143.3, 142.9, 140.0, 139.5, 109.5, 109.5, 84.2, 83.3, 58.5, 57.5, 54.6, 54.3, 37.2, 37.1), and 4 quaternary (δ127.9, 127.8, 63.0, 53.3) carbons. The appearances of both the ^1H and ^{13}C spectra resemble those of C_{15} alkaloids; most peaks appear as doublets, which suggests that this C_{30} alkaloid is a dimer with two C_{15} structural units. The ^1H–^1H COSY spectrum is consistent with the stereoisomeric compounds **(2),** known in the older literature as 6,6′-dihydroxythionuphlutine-A (Cullen et al., 1973) and thionupharodioline, and their sulfoxides. The intense ion peak at *m/e* 230 is a characteristic fragment for most *Nuphar* alkaloids; it indicates the dehydroxyl fragment of the C_{15} unit. Other fragmentations include *m/e* 216 (230 − CH_2), 262 (230 + S), and 248 (216 + S). The corresponding ions containing sulfoxide at *m/e* 278 (230 + SO) and 264 (216 + SO) are not present, indicating the isolated product is not a sulfoxide (Wrobel et al., 1972b). The two methyl carbons at δ19.1 and 19.2 (C-11 and C-11′) are assigned from the DEPT spectrum. Their chemical shifts are about 5.7 ppm downfield from that of axial methyls and thus give a clear indication of the equatorial disposition of these methyls (LaLonde and Wong, 1975). The attached protons at δ0.91 (H-11 and H-11′) are assigned from the ^1H–^{13}C COSY spectrum. Tracing the spin systems starting from the methyl protons on the ^1H–^1H COSY spectrum, the proton assignments are found to be: δ1.35 (H-1, H-1′), 1.10–1.35 (H-2, H-2′), 1.6 (H-3, H-3′), 3.6–3.7 (H-4, H-4′), 1.65 (H-8, H-8′), 1.88 (H-9, H-9′), and 2.3 (H-10, H-10′). The attached carbons are revealed from the ^1H–^{13}C COSY spectrum as: δ37.1 and 37.2 (C-1, C-1′), 32.0 and 30.3 (C-2, C-2′), 33.3 and 33.4 (C-3, C-3′), 54.3 and 54.6 (C-4, C-4′), 34.8 and 35.0 (C-8, C-8′), 28.5 and 29.1 (C-9, C-9′), and 57.5 and 58.5 (C-10, C-10′). The two hydroxyl protons were assigned to δ2.00 and 2.40 by the absence of their ^1H–^{13}C correlations in the COSY spectrum. The ^1H–^1H COSY spectrum showed the cross peaks of the hydroxyl protons and the adjacent H-6 and H-6′ protons at δ3.98 and 4.23. The corresponding carbons are δ83.3 (C-6′) and 84.2 (C-6) (Cybulski and Wrobel, 1989). Comparison of the chemical shifts of the two protons with known axial–equatorial data (Wrobel, 1977) suggests they are equatorial and the hydroxyl groups are axial. This is supported by the IR bands characteristic of the *trans*-quinolizidine ring system. The isolated compound thus cannot be thionupharodioline, which possesses equatorial hydroxyl groups on both C-6 and C-6′ and does not possess the characteristic *trans*-quinolizidine ring system bands in its IR (Cybulski and Wrobel, 1989).

The two methylene groups, thiomethylene (CH_2-17′ at δ2.20 and 2.70) and isolated methylene (CH_2-17 at δ1.80 and 1.91) are present in the ^1H–^1H COSY spectrum as isolated spin systems with geminal proton cross peaks. The corresponding carbons are assigned with C-17 at δ44.2 and C-17′ at δ39.6.

The thiolane stereochemistry with respect to the attachment of the AB and A′B′ quinolizidine ring systems has been discussed by Lalonde and Wong, whose studies were based on the structure of thiobinupharidine, thionuphlutine, and neothiobinupharidine, alkaloids having quinolizidine ring attachments at CH_2-17 of ax-ax, ax-eq, and eq-eq, respectively (LaLonde and Wong, 1977; LaLonde et al., 1974). The C-17 resonance is related to the number of axial and equatorial attachments in which the thiomethylene group is involved. The chemical shift of C-17 is gradually shifted downfield in these compounds: δ45.3, 50.6, and 54.5 ppm, respectively. With a C-17 resonance of δ44.2, our compound belongs to the thiobinupharidine stereochemical family of thiaspirane *Nuphar* alkaloids, and it is 6,6′-dihydroxythiobinupharidine **(2).**

In order to compare the activity of a known allelopathic compound in our

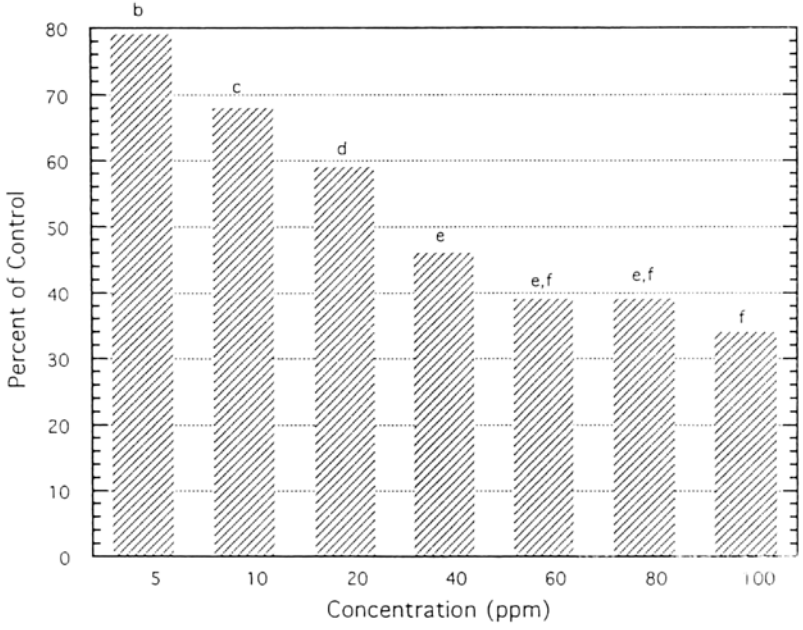

FIG. 2. Bioassay of the known allelopathic compound *dl*-2-aminoadipic acid against lettuce seedling total length (hypocotyls plus radicles). Bars with the same letter are not significantly different according to the Duncan multiple range test at $P \leq 0.05$; the control was different from all treatments.

assay system, we used commercially available *dl*-2-aminoadipic acid in our lettuce seedling bioassay at concentrations of 5–100 ppm. It exhibited progressive inhibitory effects on lettuce seedling whole-plant growth over this concentration range (Figure 2). Growth was reduced to 34% of the control value at a concentration of 100 ppm of compound.

The mixture of *N. lutea* alkaloids strongly inhibited both lettuce seedling radicles and hypocotyls; the inhibitory effect was more than 10 times that of *dl*-2-aminoadipic acid (Figure 3). Radicles appeared to be more sensitive to the alkaloid mixture than hypocotyls. At a concentration of only 6.0 ppm, the growth for radicles was 39% of the control, while for hypocotyls it was 63% of the control. At very low concentrations, 0.15–0.60 ppm, both hypocotyl and radical growth stimulation was observed, but the stimulation did not meet tests of statistical significance. Growth stimulation at low concentrations has been reported for other allelochemicals (Rice, 1984).

Nupharolutine **(1)**, solubilized in water by the addition of ethanol, showed no growth inhibitory effects toward either lettuce radicles or hypocotyls greater

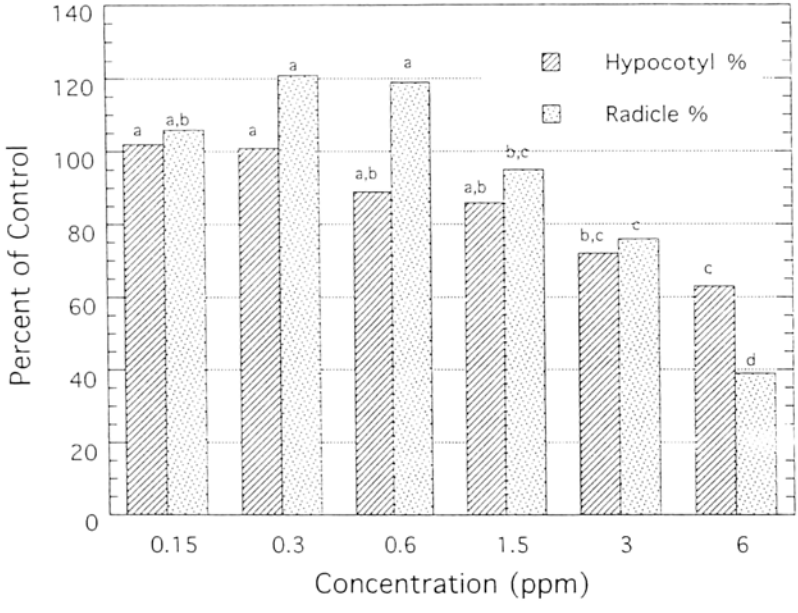

FIG. 3. Bioassay of the crude alkaloid mixture from *N. lutea* against lettuce hypocotyls and radicles. Bars with the same letter (within each assay) are not significantly different according to the Duncan multiple range test at $P \leq 0.05$. The control in the hypocotyl and radicle assays was only different from the crude alkaloid mixture at 3 and 6 ppm.

than those of the ethanol solvent over the range of 1–60 ppm. Nupharolutine is biologically inactive in this testing system. While one might predict that the more important allelopathic compounds from aquatic plants are water-soluble compounds, Fischer and Quijano (1985) described a mechanism by which water-insoluble plant compounds may act as allelochemicals and be transported via micelle formation with long-chain fatty acids.

In contrast to nupharolutine, 6,6′-dihydroxythiobinupharidine **(2)** was twice as inhibitory toward lettuce seedling growth as the crude alkaloid mixture (Figure 4). Radicle growth was reduced to 32, 27, and 17% of the control at concentrations of 1.5, 3.0, and 6.0 ppm, respectively, in one assay. A replicate assay showed no statistically significant inhibition at 1.5 ppm, but growth was reduced to 39% and 34% of the control at 3.0 and 6.0 ppm, respectively. It appears that the concentration 1.5 ppm is near the pivotal concentration between strong inhibition and no inhibition for 6,6′-dihydroxythiobinupharidine **(2).** One assay showed a significant 29% radicle growth stimulation at 0.6 ppm.

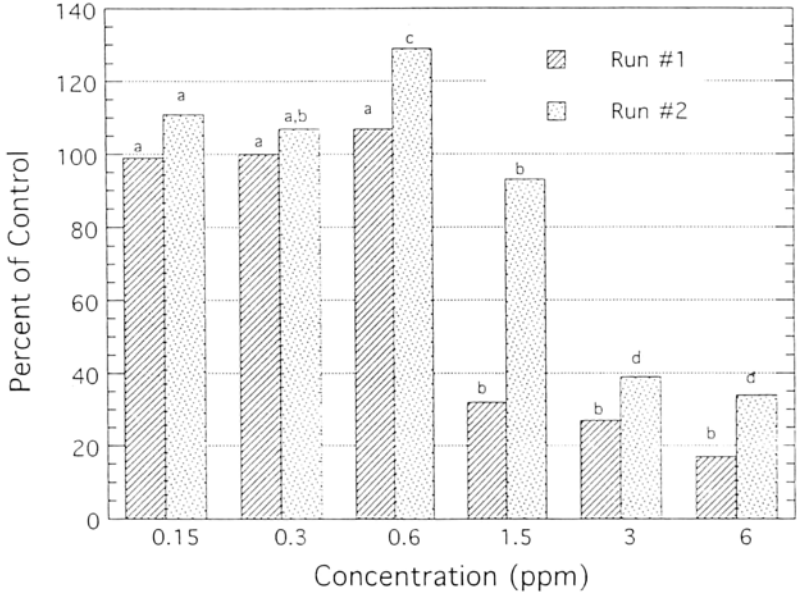

FIG. 4. The bioassay of 6,6′-dihydroxythiobinupharidine with a lettuce radicle assay. Results of replicate runs are shown. Bars with the same letter (within each run) are not significantly different according to the Duncan multiple range test at $P \leq 0.05$. The control in the lettuce radicle assay was different from the inhibitory effect of 6,6′-dihydroxythiobinupharidine at ≥ 3 ppm. At 0.6 ppm the alkaloid was significantly stimulatory in run 2.

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RESORCINOL IN EXUDATES OF *Nuphar lutea*

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Abstract—Resorcinol (1,3-dihydroxybenzene) was identified as one of the major constituents of the exudate of *Nuphar lutea* seedlings, which were raised from seeds and cultivated under axenic conditions. The compound is released from the plants in considerable amounts (up to 15 nmol/seedling/day). Highest rates of resorcinol exudation were measured when the seedlings were incubated under physiological conditions (macronutrient and light supply) that resembled those of natural stands of the plant. An inverse correlation exists between nitrate and/or light supply and resorcinol production. Because of its generally toxic properties, resorcinol is suggested to play a role as an allelochemical in interactions between macrophytes and other organisms of the aquatic ecosystem. A first approach of resorcinol application to zooplankton and phytoplankton organisms resulted in deleterious effects against a *Daphnia* species. Two Cryptophyceae species reduced resorcinol concentration to zero, showing a concomitant increase of the size of starch granule enclosures. Cyanophyceae and Chlorophyceae seemed not to be affected.

Key Words—Exudation, *Nuphar lutea*, Nymphaeaceae, yellow water lily, macrophytes, phytoplankton, zooplankton, phenols, resorcinol, allelochemicals, nitrate, light.

INTRODUCTION

Allelopathy has been suggested to participate in the formation, maintenance, distribution, and succession of terrestrial plant populations (Willis, 1994; Put-

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nam and Tang, 1986; Rice, 1984; Grodzinsky, 1982). This phenomenon implies the exudation of phytotoxins and/or pretoxins from distinct plant organs (Fischer et al., 1994) and may be considered as a defensive strategy of plants against competitors (Harborne, 1988). For aquatic plants, such mechanisms have often been postulated (Gopal and Goel, 1993; Szczepanska, 1987; Wium-Andersen, 1987; Ostrofsky and Zettler, 1986). Several workers have reported poorly defined compounds, e.g., "complex polyphenolic substances," as exudation products of marine macrophytes (Ragan and Jensen, 1979; Moebus and Johnson, 1974; Hellebust, 1974). Likewise, compounds exuded from several freshwater macrophytes could only roughly be classified as "high" (>1000 Da) or "low" (<1000 Da) molecular weight compounds (Sondergaard, 1981). A recent study, however, proved the existence of a structurally defined polyphenol, eugeniin (1,2,3-tri-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-β-D-glucose) in extracts and exudates of *Myriophyllum spicatum*, Haloragidaceae (Gross et al., 1996). In bioassays, eugeniin showed particular algicidal properties and was a potent inhibitor of algal exoenzymes, such as alkaline phosphatases (Wetzel, 1993). Among other freshwater macrophytes, *Nuphar lutea* was suspected to possess high allelopathic potential (Elakowich and Wooten, 1991). Because of good germination rates and ready availability of seedlings, this plant seemed to be ideal for laboratory experiments. Moreover, *Nuphar lutea* seedlings were recently shown to exude unknown phenolic compounds in amounts between 0.2 and 1.3 μg phenol/hr g dry weight (Sütfeld, 1993). Here we describe the structural analysis of one of the major constituents of the exudation products and its rate of exudation. A first approach was undertaken to examine the deleterious or beneficial effect of the isolate on some freshwater plankton organisms.

METHODS AND MATERIALS

Cultivation of Seedlings. Seeds of *Nuphar lutea* were harvested from natural stands and vernalized and sterilized as previously described (Sütfeld, 1993). The seeds germinated readily at 20°C. Standard incubation assays consisted of 200 seedlings (up to 16 weeks old) in 800 ml of nutrient solution. A sterile inorganic cultivation medium (Sütfeld, 1993) was modified to contain (mg/liter): Ca^{2+}: 35.0; Fe^{3+}: 0.05; K^+: 0.46; Mg^{2+}: 3.5; Na^+: 46.0; NH_4^+: 0.08; Cl^-: 61.0; HCO_3^-: 122.0; NO_3^-: 0.66; PO_4^{3-}: 0.05; SO_4^{2-}: 14.0, and a mixture of trace elements (Z8) as recommended by NIVA (1986). For special incubation assays, NO_3^- was increased to 93.0 mg/liter; in this case Na^+ was replaced by K^+ (up to 79.0 mg/liter). Cultivation and standard incubation was performed at 20°C and 20 μmol PAR/m^2/sec. Some cultures were germinated and raised in absolute darkness, whereas after germination, other cultures were grown at light intensities up to 900 μmol PAR/m^2/sec. For the latter series, temperature-stabilized

cultivation boxes, equipped with electronic energy-saving light bulbs (Dulux EL, Osram, Germany) were used.

Separation of Exudation Product. The same HPLC equipment as described Sütfeld, (1993) was used with the following alterations: Columns were 4 × 250-mm Spherisorb ODS2, 5 μm (Phase Separations Inc., Norwalk, Connecticut) or LiChrosorb RP18, 5 μm (Merck, Darmstadt, Germany), each equipped with a 4 × 30-mm precolumn (LiChrosorb RP8, 10 μm). Separations were run with H_2O as mobile phase at a flow rate that kept the pressure between 14 and 18 mPa. Aqueous samples from incubation assays were directly applied to HPLC (injection volume 1 ml, using the concentration effect of the ODS2 column); injections of ethanolic samples from compound enrichment were not allowed to exceed 60 μl to keep mobile phase constant (Sütfeld, 1989), and detection of eluted compounds occurred at 281 and 300 nm. After identification of the exudation product, resorcinol (Merck) was used for calibration. Quantitation was performed by peak integration of arbitrary units (AU_{281}). Detector response was linear in the range of 0.1–50.0 nmol/ml; resorcinol content (c) was calculated by $c = 1.79 \times AU_{281}$ (nmol/ml) ($r^2 = 0.996$, $N = 5$). The precision of peak area measurements with repeated injections of the same sample was ±0.8%; statistical analysis was performed using a Statistix version 4.0 package.

Enrichment of Exudation Product. Incubation assays were started by thoroughly rinsing the seedlings with fresh nutrient solution. The formation of the exudation product was monitored by HPLC. At an appropriate time, the nutrient solution was removed and subjected to liquid–liquid extraction: From a given total volume, 250-ml batches each were successively extracted into 300 ml of ethyl acetate (HPLC quality; Merck). The organic phase was then evaporated and the residue was dissolved in ethanol until a clear solution was obtained. Portions of 60 μl were used for HPLC; fractions containing the exudation product were collected. The combined fractions were extracted with ethyl acetate, and the organic phase was evaporated to dryness.

Structural Analysis of Exudation Product. UV spectral data were obtained during peak elution on HPLC by diode-array spectroscopy. TLC was performed on silica gel thin layer plates (Merck) with or without fluorescent indicator. For cochromatography on HPLC and TLC, catechol (1,2-dihydroxybenzene), resorcinol (1,3-dihydroxybenzene), pyrogallol (1,2,3-trihydroxybenzene), and guaiacol (1-hydroxy-2-methoxybenzene) served as reference compounds. TLC (R_f on silica gel, $CHCl_3$–HAc 2:1, water-saturated): pyrogallol 0.44; catechol 0.75; resorcinol 0.61; guaiacol 0.98; exudation product 0.61. All spots were fluorescent blue under UV_{254} and turned pale brownish 2 hr after drying in daylight. HPLC (R_t, λ_{max}): pyrogallol 5.56, 284; catechol 11.27, 284; resorcinol 12.07, 282; exudation product 12.07, 282. Guaiacol ($\lambda_{max} = 281$) did not elute under the given conditions.

^1H and ^{13}C NMR spectra were recorded on a Varian-Gemini 200 instrument

at 199.95 MHz and 50.29 MHz, respectively, in MeOH-d_4 with TMS as internal standard. Chemical shifts are given in δ (ppm). An electron impact (EI) -MS spectrum was obtained with a Finnigan MAT 312 instrument (80 eV). The GC system was a Satochrom GC; cross-linked methylsilicon capillary column (DB-1, J & W scientific) 30 m × 0.32 mm, film thickness 0.25 μm; 150°C–280°C, 3°/min, then isothermally; N_2 34 cm/sec, split 1:20, injection volume 0.6 μl, injector 250°C, FID 290°C. Resorcinol was derivatized to its TMS ether using BSTFA and TMCS. EI-MS *m/z* (rel. int. %): 110 (100), 82 (80), 81 (46), 53 (60). ^{1}H NMR: δ 6.23–6.28 (H-2, H-4, H-5, m, 3 H), 6.94 (H-5, t, 1H). ^{13}C NMR: δ 103.3 (C-2), 107.7 (C-4/C-6). 130.8 (C-5), 159.6 (C-1/C-3).

Estimates of Dry Weight and NO_3^-. Dry weight of seedlings was estimated as previously described (Sütfeld, 1993); for NO_3^- estimation the Spectroquant assay (Merck) was applied, using KNO_3 solution for calibration.

Resorcinol Application Experiments. These were performed on zooplankton and phytoplankton species that were routinely subcultured in the institute. Resorcinol (Merck) was added to a *Daphnia magna* culture (about 100 individuals/500 ml) and to several algae cutlures (about 10^5 individuals/ml) in a final concentration of 25 nmol/ml. During incubation, the resorcinol content was estimated by quantitative HPLC; the fitness of algae cultures was observed by light microscopy. Untreated cultures served as control.

RESULTS

EI-MS and NMR Data. The ^{1}H NMR spectrum of the exudation product in MeOH-d_4 exhibiting a triplet at δ 6.94 and three overlapping aromatic signals at δ 6.23–6.28 and a molecular weight of 110 (EI-MS) suggested resorcinol (1,3-dihydroxybenzene). The ^{13}C NMR spectrum was consistent with the proposed structure. All spectral data, TLC, HPLC, and a GLC of the isolate were in full agreement with authentic material.

Influence of Nitrate on Resorcinol Exudation. Under standard incubation conditions (0.66 mg NO_3^-/liter), resorcinol was, over the time range of observations, continuously being exuded from a batch of 200 seedlings with a linear production rate of approx. 3 μmol/day, resulting in a resorcinol concentration of 20 ± 1 nmol/ml within seven days. Increasing the NO_3^- content of the medium >5.0 mg/liter led to a reduced production rate. Figure 1 shows the kinetics after transferring the seedlings into nutrient solutions containing either 23 or 93 mg NO_3^-/liter. This lowered the resorcinol production rate to approx. 2 or 1 μmol/day, respectively. Under these conditions, the seedlings consumed NO_3^- at an average rate of 5 mg/day. When seedlings were preincubated for some days in medium of high (93 mg NO_3^-/liter) nitrate content, the resorcinol production dropped to zero. When these cultures were transferred into medium

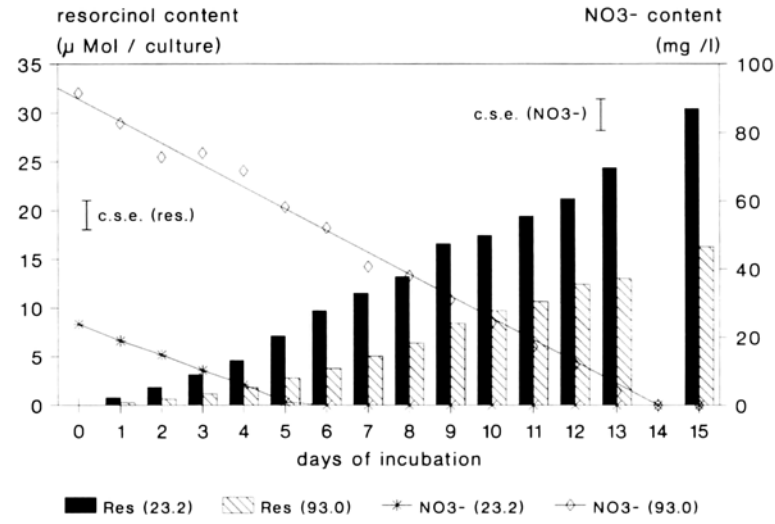

FIG. 1. Resorcinol and NO_3^- contents of nutrient solutions of *Nuphar* seedlings (preincubation in 0.66 mg/ml NO_3^- containing solution) after transfer into solutions containing 23.2 or 93.0 mg NO_3^-/liters. C.s.e., mean values ± common SE. There were five replicates per incubation series.

of lower NO_3^- content (69.9–0 mg/liter, Figure 2), resorcinol production started again, but at a remarkably reduced rate (up to 0.5 μmol/day). Under these conditions, the NO_3^- consumption was likewise reduced, averaging 0.125 mg/day.

Influence of Light on Resorcinol Exudation. When cultivated in the dark and with low (0.66 mg/liter) NO_3^- supply, seedlings of the same age produced amounts of resorcinol that were comparable to those incubated under standard conditions (20 μmol PAR/m^2 sec, Table 1). After increasing the NO_3^- concentration to 46 mg/liter, resorcinol exudation of the "dark" cultures decreased, as was observed for illuminated cultures. Under high light (900 μmol PAR/m^2 sec), resorcinol production stopped, independent of the NO_3^- supply. In contrast to resorcinol production, the amount of other compounds released [so-called "highly polar (HP) fraction"; Sütfeld, 1993] was obviously positively correlated to light supply. Under strong illumination, release of these highly polar compounds was dramatically enhanced, independent of the NO_3^- supply.

Effect of Resorcinol on Some Plankton Organisms. As shown in Table 2, resorcinol (25 nmol/ml) caused death of a *Daphnia magna* culture within five days. During this time, about 7% of the resorcinol added disappeared from the nutrient solution. No morphological effects or disappearance of resorcinol were

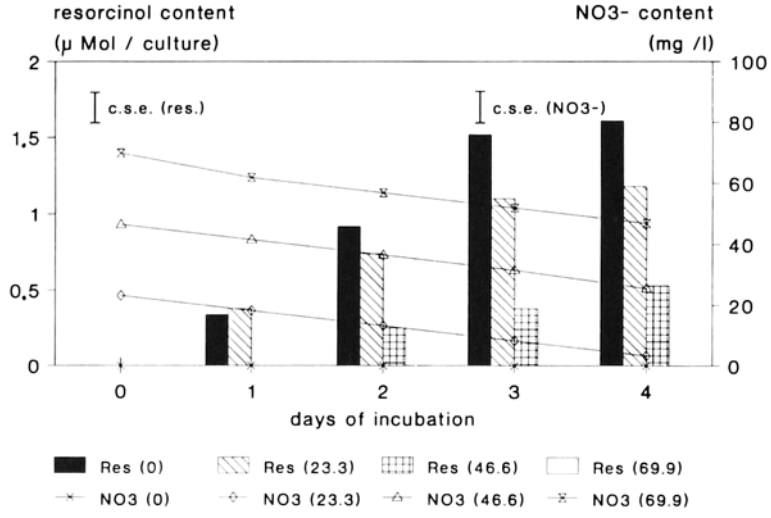

FIG. 2. Resorcinol and NO_3^- contents of nutrient solutions of *Nuphar* seedlings (preincubation in 93.0 mg/ml NO_3^- containing solution) after transfer into solutions containing 0, 23.2, 46.6, or 93.0 mg NO_3^-/liter. There were five replicates.

TABLE 1. INFLUENCE OF LIGHT AND NO_3 ON PRODUCTION OF RESORCINOL AND UNIDENTIFIED PRODUCTS (HPF)

NO_3 (mg/ml)	PAR (μmol/m^2/sec)	HPF (AU)[a]	Resorcinol produced (μmol/culture day)
0.66	20	2.38	1.04[b]
0.66	0	1.50	0.81[c]
46.0	0	3.43	0.12[c]
23.2	20	5.32	0.35
46.0	20	3.39	0
0.66	900	11.57	0
46.0	900	10.91	0

[a] HPF: highly polar fraction, R_t < 4 min; AU: arbitrary units from peak integration on HPLC.
[b] Standard incubation.
[c] Seedlings germinated and raised in absolute darkness.

TABLE 2. EFFECT OF RESORCINOL (25 nmol/ml) ON SOME PLANKTON ORGANISMS

Organism	Length of incubation (days)	Resorcinol uptaken/degraded (%)	Visible effect
Daphnia magna	5	7	death of all organisms
Chlamydomonas sp.	7	0	no effect
Scenedesmus acutus	7	0	no effect
Anabaena sp.	7	0	no effect
Cryptomonas sp.	5	100	s.g.[a]
Rhodomonas sp.	6	100	s.g.[a]

[a] Starch granules increasing in size.

observed on *Chlamydomonas*, *Scenedesmus*, or *Anabaena* cultures within seven days. When administered to *Cryptomonas* or *Rhodomonas* cultures, resorcinol disappeared completely from the nutrient solution within five or six days. During that time, a conspicuous increase in size of white compartments within the cells, supposedly starch granules, was observed.

DISCUSSION

This is, to our knowledge, the first report on the release of nonderivatized resorcinol from a higher plant and the first evidence for exudation of a structurally defined phenol from an undisturbed aquatic macrophyte. It is not known at present whether resorcinol is produced and stored unbound or whether it is liberated from a precursor, e.g., a glycoside.

1,3-Dihydroxy derivatives of benzene and more complex aromatic ring systems (resorcinylic compounds) are widespread products of plant and microbial metabolism (Harborne and Simmonds, 1964; Chapman and Ribbons, 1976). Hegnauer (1990) mentioned the occurrence of resorcinol in the heartwood of *Artocarpus* and *Morus* (Moraceae) species. Alkylated resorcinylic compounds have been reported for tissues of several *Ononis* (Leguminosae) species (Barrero et al., 1994 and references therein) and for the peel or flesh of mango fruits (Droby et al., 1987 and references therein). In the latter case, the accumulation of resorcinol derivatives is considered as a defensive response of the plants against bacterial infection. Release of 5-(12-heptadecenyl)-resorcinol in root exudates of *Oryza* plants was recently demonstrated by Bouillant et al. (1994).

We tried to adapt the physiological conditions for cultivation of *Nuphar* seedlings in the laboratory to those of the natural environment. The results of preliminary experiments showed that vernalization was necessary for germination and that germination could be induced by increasing temperature, in agreement with the findings of Smits et al. (1990) for various Nymphaeaceae. This suggests that germination of *Nuphar* seeds in natural stands takes place when the water temperature exceeds 15°C in the spring. Data for photoactive radiation, obtained from the Schöhsee lake (source of *Nuphar* used here), showed that the PAR under natural *Nuphar* stands, when measured at reasonable depths (1–2 m) on the lake bottom, averages between 5 and 20 μmol/m^2/sec (Ho, 1979; Rai, personal communication, 1995). During this season, the NO_3^- supply of the Schöhsee ranges below 5 mg/liter. This suggests that *Nuphar* seedlings, if rooted in appropriate natural places, should exude resorcinol in a manner similar to laboratory cultures.

Initially, the correlation of low light and/or low NO_3^- supply with high resorcinol production seems to be paradoxical. However, poor environmental conditions may enhance the defensive response of the plants and *vice versa*. These observations are in good agreement with those from studies of terrestrial plants that grow in arid, nutrient-limited regions. Several observations made under arid conditions suggest that nutrient limitation intensifies the allelopathic action of exuded toxins or pretoxins (Williamson et al., 1992; Fischer et al., 1994).

Resorcinol has antiseptic and membrane-affecting properties (The Merck Index, 1983), and its exudation may provide environmental benefits for *Nuphar* seedlings. In contrast to terrestrial locations, allelochemicals exuded from macrophytes into the aquatic environment may be diluted and transported more efficiently to microbes, plants, and animals of their surroundings.

A variety of microorganisms have the enzymatic equipment, i.e., catechol-1,2-oxygenase (Neujahr and Varga, 1970; Shailubhai et al., 1982; Paller et al., 1995) or catechol-2,3-oxygenase (Kachhi and Modi, 1976), to oxidize orthodihydroxybenzene derivatives such as catechol. The quinonoid intermediates produced can undergo a subsequent enzymatic ring cleavage that leads to harmless products. In addition, ortho-substituted benzene derivatives undergo spontaneous oxidation under the influence of UV light in the natural environment (Appel, 1993). However, this does not seem to occur with metadihydroxybenzenes such as resorcinol; biochemical degradation of this compound appears to be limited to a small number of microbial specialists only. Yeasts are able to metabolize resorcinol by anaerobic fermentation (Tschech and Schink, 1985), whereas other fungi degrade resorcinol by enzymatic hydroxylation. In the latter case, pyrogallol (1,2,3-trihydroxybenzene) or hydroxyquinol (1,2,4-benzenetriol) is formed as an intermediate, followed by ring cleavage (Groseclose and Ribbons, 1981; Shailubhai et al., 1983). Organisms that lack 1,3-dihydroxy-

benzene hydroxylases may accumulate resorcinol in the tissues, which may, therefore, quickly reach toxic levels.

The detection of resorcinol as an exudation product of *N. lutea* gives reason to study its ecological significance in freshwater systems. The results shown in Table 2 originated from a preliminary study in which resorcinol was applied to a zooplankton organism (*Daphnia magna*) and to several phytoplankton organisms (*Chlamydomonas, Scenedesmus, Anabaena, Cryptomonas*, and *Rhodomonas*) in a similar concentrations as produced by *Nuphar* seedlings in the previous experiments. The specificity found was surprising: A toxic effect against *Daphnia* was observed, but no effect was observed on the Chlorophyceae or the Cyanophyceae species. Cryptophyceae reduced resorcinol concentration to zero, showing a concomitant increase in the size of their starch granules. This suggests that they might have the enzymatic equipment to metabolize it. These first results suggest that resorcinol acts as an allelochemical in an aquatic medium and may be specifically directed against or beneficially used by other organisms. Investigations on these questions are in progress.

It might be argued that small amounts of an allelochemical exuded into a natural lake waterbody would have little effect on other organisms because of high dilution. Recent observations with terrestrial plants, however, suggest that a "constant flux of allelochemicals. . . , if continually taken up by target species . . . may have significant deleterious effects" (Weidenhamer et al., 1994). This is especially true for the aquatic situation.

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IDENTIFICATION OF SEX PHEROMONES FROM COWPEA WEEVIL, *Callosobruchus maculatus*, and RELATED STUDIES WITH *C. analis* (COLEOPTERA: BRUCHIDAE)[1]

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Abstract—Female cowpea weevils, *Callosobruchus maculatus*, produce a sex pheromone that elicits orientation and sexual behavior in males. Bioassay-directed isolation of the sex pheromone was conducted and compounds in the active fraction were identified and synthesized. Volatiles were collected from individual virgin females by adsorption on filter paper discs and hexane extraction. A bioassay was used in which the locomotory response of single males in glass vials was recorded upon exposure to treatments or controls. Crude extracts were subjected to silica gel column chromatography with solvents of increasing polarity; all activity eluted with methanol. Activity in the highly polar methanol fraction suggested a carboxylic acid or a compound with multiple polar functionality. Acid-base partitioning of the crude extract isolated all activity in the acid fraction, confirming that the pheromone was a carboxylic acid. The acid fraction was further fractionated by preparative GC with a Carbowax column. The most active GC fraction contained the following five 8-carbon acids identified by GC-MS and comparison with synthetic can-

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didates: 3-methyleneheptanoic acid, (*Z*)-3-methyl-3-heptenoic acid, (*E*)-3-methyl-3-heptenoic acid, (*Z*)-3-methyl-2-heptenoic acid, and (*E*)-3-methyl-2-heptenoic acid. Each of the synthetic acids was active individually for males, and combinations of two or more of the acid pheromones had an additive effect. Upwind flight responses to natural and synthetic pheromones were observed in a flight tunnel. (*Z*)-3-Methyl-2-heptenoic acid was previously identified as the sex pheromone for the related *C. analis*, but this and the other four acid pheromones from *C. maculatus* were inactive for male *C. analis*. There was no cross-attraction between *C. maculatus* and *C. analis* in reciprocal studies using extracted volatiles from females of both species. GC-MS analysis of *C. analis* female volatiles failed to detect any of the *C. maculatus* compounds but did find an unidentified C-8 acid with a GC retention time different from any of the *C. maculatus* pheromones.

Key Words—Carboxylic acids, Leguminosae, pulses, stored products, seed weevils, *Callosobruchus maculatus*, Coleoptera, cowpea weevil.

INTRODUCTION

The cowpea weevil, *Callosobruchus maculatus* (F.), is a serious cosmopolitan pest of stored dried pulses (seeds of Leguminosae). *C. maculatus* larvae feed inside dried seeds, and adults can disperse and infest pulses both in storage and in the field prior to harvest. Among major stored-product pests, *C. maculatus* remains one of the few for which a sex pheromone has not been identified for use in pest-management programs (Burkholder and Ma, 1985; Phillips, 1996). Evidence for the presence of a sex pheromone in *C. maculatus* was reported by Rup and Sharma (1978), and Qi and Burkholder (1982) documented the biology associated with female calling and sex pheromone production. The objective of the current study was to isolate and identify the sex pheromone or pheromones from female cowpea weevils. In the course of these studies, we also examined the sex pheromone of the closely related *C. analis* by performing cross-attraction experiments with collected volatiles and with synthetic pheromones.

METHODS AND MATERIALS

Insects and Collection of Volatiles. C. maculatus were maintained in laboratory cultures that originated from a commercial infestation in Madison, Wisconsin. *C. analis* were procured from a laboratory culture maintained at the USDA ARS Stored-Product Insects Research and Development Laboratory in Savannah, Georgia. Both species were reared on garbonzo beans, *Cicer arietinum* L., at 12% moisture content, 27 ± 2°C, 60 ± 5% relative humidity, with a 16 L:8 D photoperiod using methods of Qi and Burkholder (1982). Newly emerged unmated adults were separated by sex and maintained under the above

environmental conditions. Males were held singly for bioassay in capped 1.5-dr glass vials with 11.0-mm filter paper discs on the floor for footing.

Preliminary studies of air entrainment of volatiles from groups of female *C. maculatus* using adsorbents such as Porapak and Tenax (e.g., Byrne et al., 1975) resulted in extracts with relatively low pheromonal activity. Volatiles were thus collected from individual females by adsorption on 11.0-mm-diam. filter paper discs (Schleicher and Schuell, Keene, New Hampshire). Single newly emerged female beetles were placed with a host seed in corked shell vials that had a single filter paper disc on the floor and a second disk suspended 15 mm above the floor. The suspended disc was impaled on a stainless steel pin that was driven through the middle of the cork stopper of each vial. Up to 100 single-female vial preparations were set up daily and maintained for seven days under the above environmental conditions, after which the filter paper discs were removed and pooled in approximately 100 ml of HPLC-grade *n*-hexane for extraction over a minimum of 24 hr. Extracts were accumulated and stockpiled for several weeks at −20°C until processed. The quantity of pheromone in extracts was expressed as female-day equivalents (FDEs), which represents the amount of pheromone produced by a single female in one day. Thus a hexane extract of volatiles collected from 100 females over a seven-day period would contain 700 FDEs. Total extracts of 5000–30,000 FDEs were used in most isolation procedures. Prior to bioassay or purification extracts were filtered through silanized glass wool, the filter paper discs were given an additional rinsing with another 100 ml of *n*-hexane, and their volumes were reduced under a gentle stream of N_2 to a concentration of about 1.0 FDE/μl. Volatiles from female *C. analis* were collected in the same way as those from *C. maculatus*.

Bioassays. Male beetles of both species that were 3 to 8 days old were used in a simple no-choice vial bioassay (Qi and Burkholder, 1982) to detect pheromone activity during isolation and identification. The vial bioassay involved scoring the locomotory response of a single male in a 1.5-dr glass vial that was exposed to a stimulus on a suspended filter paper disc for 60 sec. Test stimuli were applied to 11.0-mm-diam. filter paper discs in 1.0 μl of hexane, or 1.0 μl of hexane only was applied as a control; 30 sec were allowed for evaporation of solvent before the discs were introduced to test vials. A test disc was attached to the end of a 11-mm-diam. aluminum rod with double-faced tape, and the rod was lowered into the male-containing vial to a point at which the disc was 1 cm above the floor of the vial. Up to four separate vial assays were conducted simultaneously so that responses to various test material and controls could be compared. Males were scored as either not responding or as demonstrating one of three levels of positive response; thus one of four response categories could be assigned. A category 1 response was scored when a male became active within 1 sec of stimulus introduction, ran on the floor of the vial, jumped or flew up to the disc, and typically remained on the disc for the 60-sec duration

of the test, during which copulation behavior was sometimes displayed. A category 2 response was when locomotion and searching occurred during the first 1–5 sec following stimulus introduction. The lowest level of positive response, category 3, was assigned when males began walking and actively searching around the bottom of the vial within 6–60 sec after introduction of the stimulus. A nonresponding male, category 4, typically remained quiescent on the bottom of the vial for the duration of the assay, however, antennal movement was noted because such materials would sometimes prove active at higher concentrations. Each experimental stimulus was assayed against 10–20 different males. Statistical analysis was not done during isolation of the pheromone, as responses were clearly all negative or all positive in one category. When comparing bioassay results of different materials that elicited positive responses, we employed Fisher's exact test (SAS, 1989) to discern differences in response.

Flight responses of 3- to 7-day-old unmated *C. maculatus* males to natural and synthetic pheromones were studied in a wind tunnel composed of 6-mm Plexiglas, very similar to one used by Bartelt et al. (1990), that was 127 cm long × 62 cm high × 61 cm deep. Flight assays were conducted between 09:30 and 14:00 hr under fluorescent lighting in a room maintained at 28°C and 50% relative humidity. Room air was drawn through the tunnel at 8.0 cm/sec, entrained to a laminar flow at the upwind end by passage through a 20-mesh stainless steel screen, and exhausted out of the room. Groups of five male beetles were assayed at a time by releasing them at the downwind end of the tunnel from a 6-cm-diam. Petri dish suspended 26 cm above the floor of the tunnel. Volatiles to be tested were presented on 11-mm-diam. filter paper discs that were impaled on a pin and suspended 26 cm above the tunnel floor at the upwind end. A 28-cm paper circle directly below the odor source on the floor of the tunnel served as a proximity target area for flying insects. Individual flight assays were conducted for 10 min, during which insects were scored for taking flight from the release point, approaching the target within 14 cm (at or past the proximity target), and contacting the source of attractant. We ran a control assay with hexane between each assay with synthetic or natural pheromones.

Instrumentation. Analytical capillary GC was performed on a Hewlett Packard 5890 gas chromatograph equipped with a splitless injector, fused silica column, and a flame ionization detector (FID). Initial column head pressure was 21 psi (1.45 kg/cm^2). Capillary GC analyses were carried out on one of two DB-1 columns (methyl silicone, 0.25-μm film thickness, bonded phase, narrow bore fused silica column, J & W Scientific); for the 30-m × 0.25-mm column, the temperature program was 60°C (1 min), 5°C/min, 210°C (30 min), and for the 50-m × 0.25 mm column, the temperature program was 45°C (1 min), 4°C/min, 200°C (30 min). Both capillary and makeup gases for capillary GC work were nitrogen.

Semipreparative scale column GC fractionation was performed on a Varian 2700 Aerograph instrument. Initial fractionation was done with a 5% OV-101

(Applied Science) on Chromosorb WAW-DMCS 100–120 mesh, 3-m × 7.5-mm-OD glass column. Isolation was ultimately carried out on a 4% Carbowax 20 M (Supelco) on chromosorb G (HP 80–100 mesh, Supelco), 6-m × 6-mm-OD glass column. Both carrier and makeup gases were nitrogen. All GC fractions were collected in glass capillary tubes (3 mm × 30 cm) using a Brownlee-Silverstein thermal gradient collector (Brownlee and Silverstein, 1968).

Mass spectra were obtained with a Finnigan 4500 automated gas chromatography/EI-CI mass spectrometer system (GC-MS). All spectra were obtained at 70 eV. NMR spectra were obtained using a Bruker AMX-300 instrument. All spectra were run in $CDCl_3$; chemical shifts are given in parts per million relative to tetramethylsilane.

Isolation and Analysis. Crude extracts of female *C. maculatus* were concentrated by fractional distillation through a Vigreaux column (17.5 in.). The concentrated extracts were chromatographed on a 150 × 7-mm-OD silica gel column (200–400 mesh, 60 Å) initially using a solvent system of 100% hexane, a mixture of 75% hexane and 25% ethyl acetate, 100% ethyl acetate, and lastly 100% methanol. The second solvent system we employed began with a 1:1 mixture of ethyl acetate and hexane, with subsequent fractions containing 5%, 10%, 20%, 40%, 80% methanol, with the last solvent being 100% methanol. Ten 1-ml fractions were collected from each solvent elution, concentrated under nitrogen, and submitted for bioassay. Results (below) suggested the pheromone was highly polar, possibly composed of alcohols or acids.

To pursue the possibility of an acid pheromone, we subjected a crude female volatiles extract to acid–base partitioning (Figure 1). The concentrated extract was treated with saturated sodium bicarbonate to yield fraction 1 and an aqueous phase. The aqueous solution was extracted with methylene chloride to

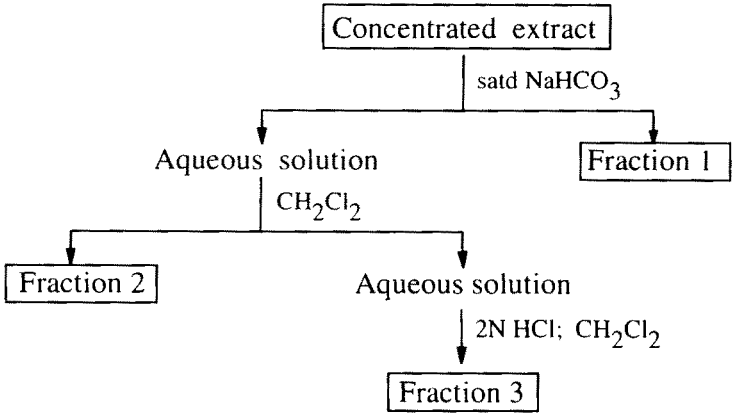


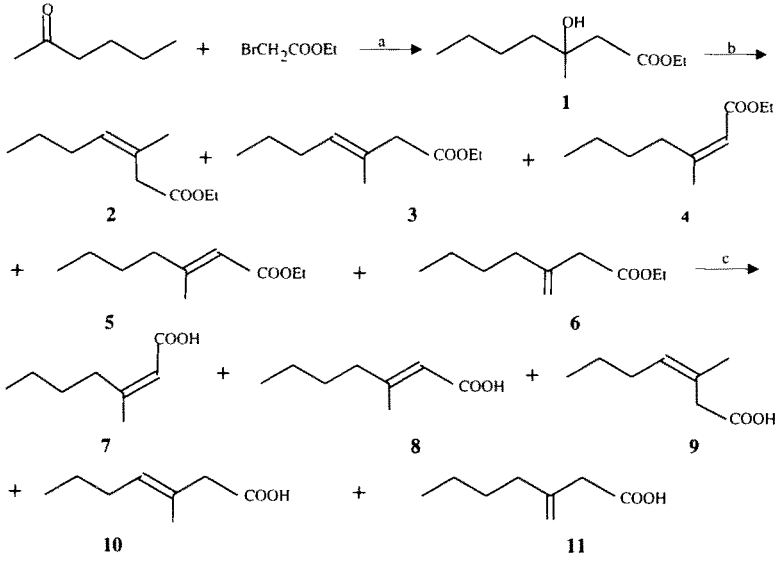
FIG. 1. Acid–base partitioning of crude volatiles extract from female *C. maculatus*.

remove polar, nonacid compounds, giving fraction 2. The remaining aqueous phase was acidified with dilute hydrochloric acid and extracted with methylene chloride to yield fraction 3. All fractions were retained for bioassay.

We used a semipreparative-scale, packed column GC fractionation on Carbowax 20 M to isolate the activity from the acidic fraction 3. The temperature program was 45°C (1 min), rising at 4°C/min to 200°C (30 min). Seven subfractions were taken at 10-min intervals and were tested in our bioassay. Because the total amount of material in the active fraction was too small for NMR experiments, no further isolation work was done.

GC-MS analysis of the active Carbowax 20 M fraction revealed a series of methyl-branched heptenoic acids. Because (*Z*)-3-methyl-2-heptenoic acid was reported as a sex pheromone of a related bruchid pest, *C. analis* (Cork et al., 1991), we undertook the synthesis of the α,β-unsaturated and the β,γ-unsaturated 3-methylheptanoic acids.

Syntheses. Two routes were employed to synthesize the branched heptenoic acids. One route was based on the Reformatsky reaction (Hauser and Breslow, 1955). Ethyl 3-hydroxy-3-methylheptanoate was made in high yield from 2-hexanone by way of the Reformatsky reaction with ethyl bromoacetate (Figure 2). Dehydration was carried out nonselectively, and the mixture of α,β- and



a. Zinc turnings, Benzene, reflux; b. $SOCl_2$, pyridine, 110 °C; c. KOH - EtOH, 70 °C.

FIG. 2. Scheme 1 for synthesis of *C. maculatus* pheromones.

β,γ-unsaturated esters was separated into individual esters by semipreparative packed column GC fractionation. Finally, the esters were saponified to give the unsaturated acids.

Ethyl 3-Hydroxy-3-methylheptanoate ***(1).*** Zinc turnings (4.6 g, 70 gatm) were added to a solution of 2-hexanone in dry benzene (30 ml). One tenth of a solution of ethyl bromoacetate (12.5 g, 75 mmol) in dry benzene (30 ml) was added to the above mixture. The mixture was warmed until the reaction started, and the remainder of the bromoester was added slowly. After addition, the reaction mixture was refluxed for 3 hr. The reaction was quenched with ammonium hydroxide. The organic layer was washed with brine and dried over anhydrous sodium sulfate. After removal of the solvent, the residue was distilled (62–64°C, 0.4 mm Hg) to yield 11.2 g (84% of a colorless liquid) of compound **1.** ^{1}H NMR (300 MHz): δ (ppm) 0.87 (t, 3 H, CH_3), 1.19 (s, 3 H, CH_3), 1.24 (t, 3 H, CH_3), 1.26–1.60 (m, 6 H, 3 CH_2), 2.36–2.49 (Dd, 2 H, CH_2COO), 3.41 (Bs, 1 H, OH), 4.10–4.17 (q, 2 H, OCH_2). ^{13}C NMR (75.5 Mhz): δ (ppm) 13.9, 14.1, 23.1, 26.1, 26.6, 41.8, 44.9, 60.4, 70.9, 172.9.

Unsaturated Esters ***(2–6).*** Freshly distilled thionyl chloride (2.24 g, 18 mmol) was added dropwise to a solution of compound **1** (2.82 g, 15 mmol) in pyridine with cooling by an ice bath. After the addition was complete, the reaction mixture was heated to 110°C for 1 hr. The mixture was diluted with ether, washed with dilute hydrochloric acid, and the organic phase was dried over anhydrous sodium sulfate. After removal of the solvent, the dark residue was decolorized by elution through a short silica gel column (270 mm × 21 mm OD) with 15% ethyl acetate in hexane. After removal of the solvent, the residue was distilled (85–95°C, 2 mm Hg) to yield 1.38 g (54%) of mixed unsaturated esters **(2–6).** The mixture was subjected to semipreparative-scale packed column GC fractionation to separate pure individual esters. The temperature program was 80°C (10 min), 4°C/min, 230°C (30 min). The NMR data for the unsaturated esters are given below.

The configuration of compounds **4** and **5** were determined from the chemical shifts of methyl groups attached to the carbon–carbon double bond (Silverstein et al., 1991). The chemical shift of the methyl group of compound **4** was at δ ppm 1.86, which corresponded to the *Z* configuration, while that of compound **5** was at δ ppm 2.13, which corresponded to the *E* configuration. The configurations of compounds **2** and **3** were assigned by comparison of their ^{1}H NMR spectra with literature spectra of related 3-unsaturated acids (Cork et al., 1991) and esters (Camps et al., 1987).

Ethyl (3Z)-3-Methyl-3-heptenoate ***(2).*** ^{1}H NMR (300 MHz): δ (ppm) 0.89 (t 3 H, CH_3), 1.18–1.44 (m 5 H, CH_2, CH_3), 1.78 (q, 3 H, $=CCH_3$), 1.94–2.04 (Dq, 2 H, $CH_2C=$), 3.03 (s, 2 H, $=CCH_2COO$), 4.08–4.17 (q, 2H, $COOCH_2$), 5.34 (Dt, 1 H, =CH). ^{13}C NMR (75.5 MHz): δ (ppm) 13.7, 14.1, 22.7, 23.8, 30.2, 37.6, 60.4, 128.0, 129.0, 171.5.

Ethyl (3E)-3-Methyl-3-heptenoate **(3).** ^{1}H NMR (300 MHz): δ (ppm) 0.89 (t, 3 H, CH_3), 1.18–1.44 (m, 5 H, CH_2, CH_3), 1.68 (s, 3 H, $=CCH_3$), 1.96–2.05 (q, 2 H, $CH_2C=$), 2.97 (s, 2 H, $=CCH_2COO$), 4.13 (q, 2 H, $COOCH_2$), 5.28 (Dt, 1 H, =CH).

Ethyl (2Z)-3-Methyl-2-heptenoate ***(4).*** ^{1}H NMR (300 MHz): δ (ppm) 0.91 (t, 3H, CH_3), 1.23–1.50 (m, 7 H, 2 CH_2, CH_3), 1.86 (q, 3 H, $=CCH_3$), 2.61 (t, 2H, $CH_2C=$), 4.08–4.17 (q, 2 H, $COOCH_2$), 5.63 (m, 1 H, =CH). ^{13}C NMR (75.5 MHz): δ (ppm) 13.9, 14.3, 22.8, 25.1, 30.3, 33.1, 59.3, 116.1, 160.5, 166.3.

Ethyl (2E)-3-Methyl-2-heptenoate ***(5).*** ^{1}H NMR (300 MHz): δ (ppm) 0.89 (t, 3 H, CH_3), 1.22–1.49 (m, 7H, 2 CH_2, CH_3), 2.11 (t, 2 H, $=CCH_2$), 2.13 (s, 3 H, $=CCH_3$), 4.07–4.16 (q, 2 H, $COOCH_2$), 5.63 (t, 1 H, =CH). ^{13}C NMR (75.5 MHz): δ (ppm) 13.7, 14.2, 18.6, 22.2, 29.5, 40.6, 59.3, 115.5, 160.0, 166.8.

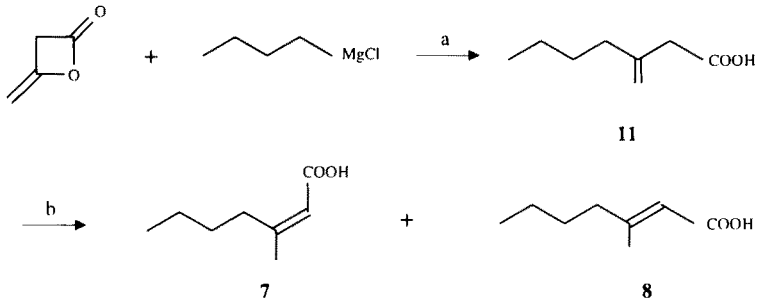
Ethyl 3-Methyleneheptanoate ***(6).*** ^{1}H NMR (300 MHz): δ (ppm) 0.90 (t, 3 H, CH_3), 1.21 (t, 3 H, CH_3) 1.25–1.49 (m, 4 H, 2 CH_2), 2.11 (t, 2 H, $=CCH_2$), 3.03 (s, 2 H, $=CCH_2COO$), 4.14 (q, 2 H, $COOCH_2$), 4.90 (Dd, 2 H, $=CH_2$).

Unsaturated Acids ***(7–11).*** Potassium hydroxide (0.98 g) was dissolved in water (2.1 ml) and diluted with EtOH (6 ml). Each isolated ester (**2–6,** the amount of each ranged from 5 to 100 mg) was dissolved in 1 ml of the KOH solution. The reaction mixture was kept at 70°C for 4 hr. After removal of the ethanol by a nitrogen stream, water was added to the residue and the mixture was extracted with ether. The remaining aqueous solution was acidified with 3 N HCl and extracted with methylene chloride. The extracts were washed with brine and dried over anhydrous sodium sulfate. After removing the solvent, acids **9** and **10** were isolated in a highly pure state. NMR data are given below.

(3Z)-3-Methyl-3-heptenoic Acid ***(9).*** ^{1}H NMR (300 MHz): δ (ppm) 0.89 (t, 3 H, CH_3), 1.24–1.44 (m, 2 H CH_2), 1.81 (q, 3 H, $=CCH_3$), 1.99 (Dq, 2 H, $=CCH_2$), 3.08 (s, 2 H, $=CCH_2COO$), 4.94 (m, 1 H, =CH). ^{13}C NMR (75.5 MHz): δ (ppm) 13.7, 15.2, 22.7, 30.3, 37.1, 127.4, 129.7, 177.1.

(3E)-3-Methyl-3-heptenoic Acid ***(10).*** ^{1}H NMR (300 MHz): δ (ppm) 0.90 (t, 3 H, CH_3), 1.20–1.45 (m, 2 H, CH_2), 1.71 (d, 3 H, $=CCH_3$), 2.02 (q, 2 H, $=CCH_2$), 3.02 (s, 2 H, $=CCH_2COO$), 5.29 (s, 1 H, =CH). ^{13}C NMR (75.5 MHz): δ (ppm) 13.7, 16.2, 22.6, 30.1, 44.7, 127.7, 130.1, 178.0.

All five acids showed strong biological activity, but compound **11** could not be isolated in high purity because the amount available from the above procedure was very limited. Furthermore, acids **7** and **8** were slightly contaminated. In order to obtain pure acid **11,** another synthetic route was employed (Figure 3) based on the addition of a Grignard reagent to diketene in the presence of cobalt (Fujisawa et al., 1982). The resulting 3-methyleneheptanoic acid was obtained free of the other isomers. This compound could be cleanly rearranged



a. CoI_2, ether, -78 °C, 5 hr.; b. TsCl-pyridine, 30 °C, 1.5 hr..

FIG. 3. Scheme 2 for synthesis of *C. maculatus* pheromones.

to a mixture of 2*Z* and 2*E* heptenoic acids (ratio 1:2) by treatment with toluenesulfonyl chloride (TsCl) in pyridine. The mixture was separated to give pure compounds **7** and **8** by semipreparative packed column GC fractionation as described above.

3-Methyleneheptanoic Acid ***(11)***. Diketene (8.07 g, 96 mmol) was added to a solution of cobalt iodide (3.40 g, 10.9 mmol) in ether (216 ml) at −78°C under a nitrogen atmosphere. *n*-Butylmagnesium chloride (2.0 M in ether, 60 ml) was slowly added dropwise and stirring was continued at −78°C for 5 hr. The reaction was quenched with 6 N HCl, and the aqueous phase was extracted with ether. The extracts were washed with brine and dried over anhydrous sodium sulfate. Distillation (141–146°C, 33 mm Hg) gave 5.2 g (38%) of compound **11** as a colorless liquid. ^{1}H NMR (300 MHz): δ (ppm) 0.93 (t, 3 H, CH_3), 1.29–1.47 (m, 4 H, 2 CH_2), 2.13 (t, 2 H, $=CCH_2$), 3.08 (s, 2 H, $=CCH_2COO$), 4.93 (s, 1 H, =CH), 4.96 (d, 1 H, =CH). ^{13}C NMR (75.5 MHz): δ (ppm) 13.9, 22.3, 29.6, 35.6, 41.6, 114.0, 142.0, 178.0.

(2Z)-3-Methyl-2-heptenoic Acid ***(7)***. TsCl (2.86 g, 15 mM) was dissolved in 50 ml of pyridine, and the solution was cooled to 0°C. Compound **11** (1.50 g, 0.5 mM) was added to the above solution. After the addition, the reaction mixture was warmed to 30°C and stirred for 1.5 hr. The reaction was quenched with water (50 ml) and stirred for 0.5 hr. The mixture was poured into cold HCl (6 N, 50 ml) and extracted with ether. The ethereal solution was treated with 10% NaOH. The alkaline solution was acidified with 6 N HCl and extracted with ether. The extracts were washed with brine and dried over anhydrous sodium sulfate. After the solvent was removed, the unsaturated acids **7** and **8** were isolated by packed column GC fractionation. The NMR data for compound **7** were: ^{1}H NMR (300 MHz): δ (ppm) 0.92 (t, 3 H, CH_3), 1.18–1.51 (m, 4 H,

2 CH_2), 1.92 (s, 3 H, $=CCH_3$), 2.14 (t, 2 H, $=CCH_2$), 5.69 (s, 1 H, =CH). ^{13}C NMR (75.5 MHz): δ (ppm) 13.9, 22.8, 25.4, 30.3, 115.0, 164.0, 170.1.

(2E)-3-Methyl-2-heptenoic Acid (8). ^1H NMR (300 MHz): δ (ppm) 0.92 (t, 3 H, CH_3), 1.18–1.51 (m, 4 H, 2 CH_2), 2.12 (t, 2 H, $=CCH_2$), 2.16 (s, 3 H, $=CCH_3$), 5.70 (s, 1 H, =CH). ^{13}C NMR (75.5 MHz): δ (ppm) 13.9, 22.3, 29.5, 40.9, 114.8, 163.7, 169.0.

RESULTS

Hexane extracts of filter paper-adsorbed volatiles from female *C. maculatus* were consistently very active when tested against conspecific males in single vial bioassays. Typically, greater than 90% of males responded positively to 1 FDE of extract within 5 sec of exposure (response categories 1 and 2). When a dilution series of a particular extract of female *C. maculatus* volatiles was bioassayed against males, the concentration that elicited 50% positive response within the 1-min assay (response categories 1, 2 or 3) was 0.0275 FDE. Male responses diminished to levels similar to that elicited by solvent controls (12% in response category 3, 88% with no response) at female volatile concentrations less than 0.00172 FDE.

Initial fractionation of *C. maculatus* volatiles by semipreparative GC using OV-101 revealed activity in two broad areas of retention corresponding to compounds of C_8 and larger. Combination of these two GC fractions yielded activity equivalent to that elicited by either of the fractions presented singly. When each of the two active fractions was further subfractionated on OV-101, each subfraction was attractive to *C. maculatus* males. The similar levels of activity in numerous contiguous GC fractions suggested tailing or peak broadening of at least one compound, or the presence of a series of structurally similar compounds, all with pheromone activity. Analytical capillary GC and GC-MS of the most active subfractions from OV-101 were inconclusive and did not contain adequate amounts of material for analysis.

Polar gradient silica gel chromatography of *C. maculatus* female volatiles failed to isolate any substantial activity in fractions with hexane, a 4:1 mixture of hexane + ethyl acetate, and pure ethyl acetate (Table 1). Concentration of fractions 17, 18, and 19 to 1/10th their initial volumes revealed activity in the 25–50% response levels, suggesting some limited activity in these polar fractions. Recombination of the original 20 silica gel fractions lacked activity, which indicated that the majority of the pheromone must have remained on the column. Final elution of the column with methanol yielded a fraction that elicited 100% response of male *C. maculatus* within 5 sec in the single vial bioassay (Table 1, fraction 21). A second silica gel chromatography experiment that used increasing concentrations of methanol in a 1:1 mixture of hexane and ethyl

TABLE 1. BIOASSAY RESPONSES OF *C. maculatus* MALES TO SILICA GEL CHROMATOGRAPHY FRACTIONS OF *C. maculatus* FEMALE VOLATILES

Fraction description[a]	Response % in Four Categories[b]			
	1	2	3	4
Solvent control (hexane)	0	0	20	80
Hexane, nos. 1-10	0	0	7	93
Hexane-Ethyl acetate (4:1), nos. 11-19	0	0	20	80
Ethyl acetate, no. 20	0	0	40	60
Combination of fractions 1-20	0	0	20	80
Methanol, no. 21	75	25	0	0
Original sample (unfractionated)	100	0	0	0

[a] Numbered fractions are sequential collections from a silica gel column; 10-20 males were bioassayed against each fraction.
[b] Categories 1-3 are positive responses and category 4 is no response; see text for details.

acetate found all pheromone activity in the 80% and 100% methanol fractions. Isolation of activity predominantly in the methanol fractions suggested the pheromone was a carboxylic acid. Acid-base partitioning of concentrated whole extract confirmed that the pheromone was a carboxylic acid. Pheromone activity was found only in the reacidified organic acid fraction (fraction 3, Figure 1), and no activity was detected in the deacidified organic fraction (fraction 2) or in the nonacid aqueous phase (fraction 1). Acid-base partitioning of larger hexane extracts of *C. maculatus* female volatiles was thus performed prior to isolation and identification of the pheromone.

The acid fraction of a 32,000-FDE extract of *C. maculatus* female volatiles was subfractionated on GC using Carbowax 20 M, and the last three subfractions of seven were biologically active in single-vial bioassays. Subfraction 6, which had the strongest activity in the bioassay, was analyzed by both EI and CI GC-MS. This analysis revealed five compounds that had suggested structures of methyl-branched heptenoic acids based on fragmentation and tentative matches from the GC-MS spectral library. Because of our GC-MS data and the earlier report of a methylheptenoic acid pheromone in a related species (Cork et al., 1991), we pursued the syntheses of methyl heptenoic acids (above). Comparisons of spectra and GC retention times of synthesized saturated and monounsaturated methyl heptanoic acids with those of the five compounds isolated in subfraction 6 confirmed the identification of the following acids: (3*Z*)-3-methyl-3-heptenoic acid **(9)**, (3*E*)-3-methyl-3-heptenoic acid **(10)**, 3-methyleneheptanoic acid **(11)**, (2*Z*)-3-methyl-2-heptenoic acid **(7)**, and (2*E*)-3-methyl-2-heptenoic acid **(8)**.

Both single-vial and wind-tunnel bioassays showed that all five of the identified acids were active for male *C. maculatus*. In single-vial assays each of the five compounds elicited 75% or greater responses within 5 sec (response categories 1 and 2) when tested at the 100 ng level, and 60% or greater (except for the 2 *Z* acid) when tested at the 10 ng level (Table 2). Comparison of activities among the five acids and a five-way combination found that the 3*Z* and 3*E* compounds elicited the highest responses, and these were similar to the response elicited by the combination. In wind-tunnel bioassays with natural extracts containing female pheromone, all male *C. maculatus* initiated upwind flight, 60–85% of these flew to within the 14-cm radius surrounding the source, and 40–50% contacted the source of pheromone (Figure 4). When 200 ng of each of the five synthetic acids were bioassayed, all compounds elicited upwind flight, but beetles approached and contacted the source only in response to the methylene, 3*Z* and 3*E* acids, and to the five-acid combination.

TABLE 2. RESPONSES OF *C. maculatus* MALES TO FIVE SYNTHETIC CARBOXYLIC ACIDS IDENTIFIED FROM ACTIVE FRACTION OF FEMALE *C. maculatus* VOLATILES

Compound and amount tested[a]	Response in four categories[b]				Diff[c]
	1	2	3	4	
100 ng each					
Methylene heptanoic acid	8.3	75.0	0	16.1	B
3*Z*	33.3	58.3	8.3	0	AB
3*E*	66.6	33.3	0	0	A
2*Z*	16.6	58.3	8.3	16.6	B
2*E*	8.3	83.3	0	8.3	B
5-Way combination (20 ng ea.)	66.6	33.3	0	0	A
Control (hexane)	0	0	0	100	C
10 ng each					
Methylene heptanoic acid	16.6	50.0	16.6	16.6	AB
3*Z*	0	75.0	8.3	16.6	A
3*E*	8.3	75.0	16.6	0	A
2*Z*	8.3	25.0	25.0	41.6	B
2*E*	41.6	16.6	25.0	16.6	B
5-Way combination (20 ng ea.)	16.6	66.6	16.6	0	A
Control (hexane)	0	0	8.3	91.6	C

[a] A minimum of 12 males were bioassayed for each treatment and control.
[b] Categories 1–3 are positive responses and category 4 is no response; see text for details.
[c] Distribution of categorical responses to treatments within a group followed by different letters are significantly different ($P < 0.05$; 4-way Fisher's exact test, to determine the probability that a greater deviation between two data sets exists, was performed on all pairwise combinations).

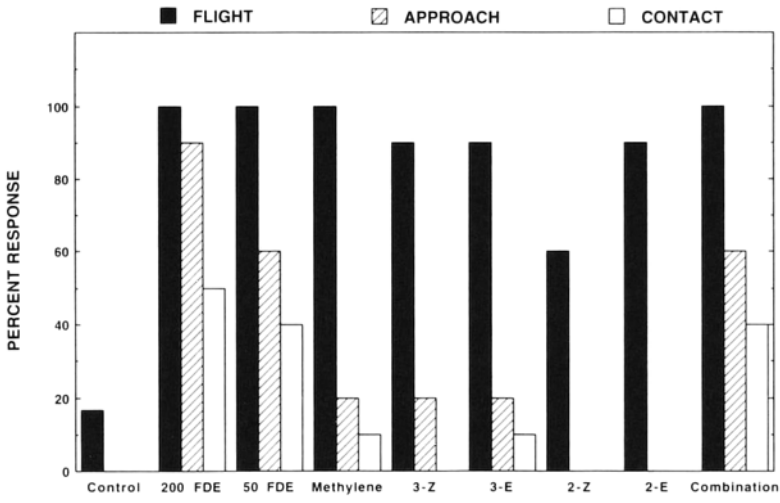


FIG. 4. Responses of male *C. maculatus* in a wind tunnel to natural and synthetic pheromone of females. Histogram bars represent the percentage of males taking flight from the release point (solid), approaching the odor source within a 14-cm radius (hatched), and those making contact with the odor source (open). Treatments were extracts of female volatiles (at 200 and 50 FDE), 200 ng of each of the five synthetic acids discussed in the text, and 200 ng of a combination of the five acids (40 ng each); control was a solvent blank.

Behavioral and chemical studies with *C. maculatus* and *C. analis* proved that the two species were not cross-attractive and that none of the five acids produced by female *C. maculatus* served as a sex pheromone for *C. analis*. In single-vial bioassays males of each species responded best to volatiles collected from conspecific females, and in most cases did not respond to volatiles from the other species (Table 3). Responses of *C. analis* males to the five synthetic *C. maculatus* acids in single vial bioassays did not differ from responses to solvent controls, except for the 2Z acid in one experiment (Table 4). Acid–base partitioning (Figure 1) of an extract of *C. analis* female volatiles confirmed via bioassay that the pheromone for this species was composed of one or more carboxylic acids. GC-MS analysis of the concentrated acid fraction from *C. analis* females, using both full-scan and single-ion monitoring modes, failed to detect any of the five acids identified from *C. maculatus* females. However, a single compound with retention time earlier than the five *C. maculatus* acids was detected, and it had a mass spectrum suggestive of a methylheptenoic acid. Identification of this compound was not pursued in the current project.

TABLE 3. RESPONSES OF *C. maculatus* AND *C. analis* MALES TO VOLATILES FROM CONSPECIFIC AND INTERSPECIFIC FEMALES

Male tested[a]	Volatile source/amt.	Response % in four categories[b] 1	2	3	4	Prob.[c]
C. maculatus	*C. maculatus* 1.0 FDE	83.3	8.3	0	8.3	0.000
	Solvent control	0	0	25.0	75.0	
	C. analis 1.4 FDE	0	6.6	46.6	46.6	0.264
	Solvent control	0	0	26.6	73.3	
	C. analis 5 FDE	0	33.3	46.6	20.0	0.004
	C. analis 10 FDE	0	6.6	40.0	53.3	0.450
	Solvent control	0	0	26.6	73.3	
C. analis	*C. analis* 1.4 FDE	20.0	66.6	6.6	6.6	0.000
	Solvent control	0	0	33.3	66.6	
	C. maculatus 1.4 FDE	0	6.6	20.0	73.3	0.100
	Solvent control	0	0	0	100.0	
	C. maculatus 5 FDE	0	0	60.0	40.0	0.139
	C. maculatus 10 FDE	0	13.3	46.6	20.0	0.181
	Solvent control	0	0	26.6	73.3	

[a] At least 12 males were bioassayed for each treatment and control.
[b] Categories 1–3 are positive responses and category 4 is no response; see text for details.
[c] Fisher's exact test between each treatment and control within a group; value given is the probability that a greater deviation between data sets could occur.

DISCUSSION

This study clearly shows that the five identified carboxylic acids are produced by female *C. maculatus* and that they elicit long-range orientation by males. The five acids apparently elicit behavioral responses in an additive manner, since a combination of the five was similar in activity to equivalent amounts of the individual compounds. Vial and wind-tunnel bioassays with synthetic compounds suggest that the 3*Z* and 3*E* acids have greater biological activity than the other acids, but further work is needed. Because these compounds elicit flight response by male *C. maculatus*, we are confident that they could serve as lures for traps to monitor populations of *C. maculatus* in pest management programs.

The sex pheromone of *C. maculatus* is one of only a few sex pheromone systems that have been described for bruchid beetles. Horler (1970) identified an ester from males of *Acanthoscelides obtectus*, but the biological evidence for this specific material serving as a pheromone is limited (e.g., Halstead, 1973). Although Cork et al. (1991) identified compound **7**, (2*Z*)-3-methyl-2-heptenoic

TABLE 4. RESPONSES OF *C. analis* MALES TO FIVE SYNTHETIC ACIDS IDENTIFIED AS SEX PHEROMONE FOR *C. maculatus*

Compound and amount tested[a]	Response % in four categories[b]				
	1	2	3	4	Diff[c]
200 ng each					
Methylene heptanoic acid	0	8.3	0	91.6	A
3*Z*	0	18.2	0	81.8	A
3*E*	0	33.3	16.6	50.0	A
2*Z*	0	33.3	16.6	50.0	A
2*E*	0	8.3	8.3	83.3	A
Solvent control	0	8.3	8.3	83.3	A
100 ng each					
Methylene heptanoic acid	0	8.3	41.6	50.0	AB
3*Z*	0	8.3	50.0	41.6	AB
3*E*	0	25.0	16.6	58.3	AB
2*Z*	0	33.3	33.3	33.3	A
2*E*	0	33.3	16.6	50.0	AB
Solvent control	0	0	16.6	83.3	B
100 ng 2*Z*	0	16.6	16.6	66.6	A
100 ng 2*E*	0	0	16.6	83.3	A
50 ng ea. 2*Z* + 2*E*	0	16.6	16.6	66.6	A
Solvent control	0	0	25.0	75.0	A

[a] At least 12 males were bioassayed for each treatment and control.
[b] Categories 1–3 are positive responses and category 4 is no response; see text for details.
[c] Distribution of categorical responses to treatments within a group followed by different letters are significantly different ($P < 0.05$; 4-way Fisher's exact test, to determine the probability that a greater deviation between two data sets exists, was performed on all pairwise combinations).

acid, as a single pheromone produced by female *C. analis*, our results do not support their conclusion. Either compound **7** occurs in female *C. analis* at levels below our detection limits, or the pheromone of *C. analis* is composed of one or more acids structurally similar to **7**. Tanaka et al. (1981) reported a female-produced pheromone for *C. chinensis* L. that is composed of a series of hydrocarbons and a dicarboxylic acid; none of the individual compounds were active alone, and activity was demonstrated only when the dicarboxylic acid was presented with the hydrocarbon mix. The pheromone system we report here for *C. maculatus* is based on acids, and does not require synergism by hydrocarbons. The *C. chinensis* work described both short-range attraction and copulation behavior elicited by the sex pheromone (Tanaka et al. 1981; Yamamoto 1990). Although we commonly observed copulation behavior in male *C. maculatus* exposed to natural or synthetic pheromone in vial bioassays, the flight responses

we observed in the wind tunnel indicate these acid pheromones operate as long-range sex attractants.

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THE PHEROMONE OF THE EASTERN TENT CATERPILLAR, *Malacosoma americanum* (F.) (LEPIDOPTERA, LASIOCAMPIDAE)[1]

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Abstract—The pheromone system of the eastern tent caterpillar, *M. americanum*, has been identified as a mixture of (*E,Z*)-5,7-dodecadienal and the corresponding alcohol. Field data on the attractiveness of the aldehyde alone were not consistent, but mixtures of aldehyde and alcohol in varying proportions were attractive to males. Addition of small amounts of *E,Z* acetate to *E,Z* aldehyde had no effect on male response, but larger amounts reduced trap catch. Traps baited with *Z,E*, *E,E*, or *Z,Z* aldehydes were not more attractive than blank traps. Pherocon 1C traps fortified with extra adhesive and baited with lures consisting of 500 μg (*E,Z*)-5,7-dodecadienal with either 250 or 100 μg of the corresponding alcohol trapped as many as 100 males/trap/night with means of 15–20. Lures prepared from purified (94% *E,Z*) aldehyde and alcohol were more attractive than those prepared from unpurified (58% *E,Z*) materials.

Key Words—Attractant, reproductive isolation, field trapping, (*E,Z*)-5,7-dodecadienal, (*E,Z*)-5,7-dodecadien-1-ol, eastern tent caterpillar, *Malacosoma americanum*, sex pheromone.

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[1] Mention of a company name or proprietary product does not constitute an endorsement by the U.S. Department of Agriculture.

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INTRODUCTION

Tent caterpillars of the genus *Malacosoma* are widely distributed over the northern hemisphere and are particularly common in temperate North America (Stehr and Cook, 1968; Fitzgerald, 1995). The forest tent caterpillar (*Malacosoma disstria* Hübner) is the most important economically, being the major deciduous forest defoliator in parts of Canada (Myers, 1993). Its pheromone was identified as a mixture of (*Z*,*E*)-5,7-dodecadienal (*Z*5,*E*7-12 : Ald) and the corresponding alcohol *Z*5,*E*7-12 : OH (Chisholm *et al.*, 1980). Field trapping confirmed these compounds as being attractive to *M. disstria* males in the field, and subsequent studies from the same group (Chisholm *et al.*, 1982) alluded to the fact that these two compounds plus (*Z*)-7-dodecenal were more attractive than virgin females, but details have not been published. The use of *Z*,*E* aldehyde for communication disruption was successful in the field (Palaniswamy *et al.*, 1983) as was use of the *Z*,*E* or *Z*,*Z* diene aldehydes, the *Z*7 monoene aldehyde, or a crude Wittig reaction product isomer mixture of 5,7-dodecadienals (Chisholm *et al.*, 1982). The pheromone of the western tent caterpillar (*M. californicum* Packard) was reported to contain *E*5,*Z*7-12 : Ald as "a component" on the basis of electroantennogram studies, field trapping, and gas chromatography–mass spectrometry (Underhill *et al.*, 1980). Early reports on the Palearctic species *M. neustrium* L. suggested the presence of *Z*5-12 : OH in the pheromone (Melikyan *et al.*, 1980). Subsequent studies did not confirm these components, but instead showed the presence of *Z*5,*E*7-12 : Ald, *E*5,*Z*7-12 : Ald, *Z*5,*E*7-12 : OH, *E*5,*Z*7-12 : OH, *Z*5,*E*7-12 : OAc, and *E*5,*Z*7-12 : OAc in ratio of 0.3 : 1.6 : 0.1 : 1.0 : 0.8 : 0.6 (Konyukhov and Kovalev, 1988). The *EZ* aldehyde or a 3 : 1 mixture of the *EZ* aldehyde and *EZ* alcohol was most attractive in the field.

The eastern tent caterpillar, *M. americanum* (F.), is a widespread inhabitant of eastern North America, usually attacking wild cherry but occasionally a pest on apples, crabapples, or various cultivated *Prunus* spp. We investigated the pheromone of this species for purposes of developing a lure for monitoring and investigating the characteristics of the reproductive barrier between the sympatric *M. americanum* and *M. disstria*. Although a preliminary report on this pheromone has been made (Hill and Roelofs, 1980), we continued our investigations to improve the lures for use in the field.

METHODS AND MATERIALS

Insects. Late instars were collected from black cherry (*Prunus serotina* L.) in the vicinity of Geneva, NY, and reared to pupation in the laboratory on host foliage in growth chambers at 20°C with a photoperiod of 16L : 8D. Females

in Maryland were also collected as late instars from *P. serotina* around Beltsville, MD, and reared to pupation on host foliage in a greenhouse under ambient conditions.

The last two abdominal segments (female tips) were removed from adult female moths during their first scotophase and extracted with Skellysolve B. Airborne collections of female effluvia were carried out as described by Hill *et al.* (1979). Laboratory compressed air was purified by passage through a precolumn of Porapak Q (1.8 × 6 cm), humidified by passage over distilled water, drawn through a glass vessel containing the female moths, and through a Porapak Q collection column (1.8 × 13 cm). Air flow of 1–2 liters/min was maintained by a model 3 Dyna pump. A second outlet from the humidifier served to release excess air. Redistilled methylene chloride was used to elute collected materials from the Porapak.

Gas Chromatography. Gas chromatography (GC) was carried out on a Packard model 7400 instrument. Glass tubing columns (2 m × 4 mm) were packed with 3% OV-1 or OV-101 on 100–120 mesh Gas-Chrom Q or 3% CHDMS (Hi-Eff 8BP, cyclohexanedimethanol succinate) on 100–120 Gas-Chrom Q. Nitrogen was used as carrier gas, and the instrument was equipped with a flame ionization detector. Materials were collected as 1-min fractions in 30-cm glass capillary tubes by connecting the capillary to the outlet of the column with a short piece of Teflon capillary tubing and placing a piece of Dry Ice on top of the capillary.

EAG Tests. Electroantennogram (EAG) studies were carried out by our standard methods as described in the review by Roelofs (1984). Briefly, male antennae were removed, placed in sticky wax in a dish containing insect saline, and connected between chloridized silver electrodes. Test materials, either standards or natural materials collected from the GC, were introduced by puffing 1 ml of air through a disposable pipet containing a standard on a small piece of filter paper, or a capillary tube containing material from the GC, into an airstream passing over the antenna.The electrical response, after amplification, was displayed on a storage oscilloscope. For natural materials, antennal response was plotted versus retention time, and standards were plotted as response versus double-bond position and configuration.

Synthetic Pheromones. Chemicals used in the field studies in Geneva, NY, were obtained from Zoecon Corporation (Palo Alto, CA) (alcohols) or were prepared from the Zoecon alcohols with pyridinium chlorochromate (aldehydes) (Corey and Suggs, 1975) or acetyl chloride–pyridine (acetates). In 1988, *EZ* alcohol was obtained from Darwin Reed (National Research Council, Saskatoon, Saskatchewan, Canada). For the later Maryland studies, *EZ* alcohol was prepared by the method of Chisholm *et al.* (1981), converted to the acetate, and purified by high-pressure liquid chromatography (HPLC) on a silver nitrate–silica gel column (20% $AgNO_3$ on 10 μm Spherisorb, 2 columns in series, 10

mm × 25 cm, toluene eluent at 6 ml min). The crude material in 1994 had a composition of 58% *E,Z*, 38% *E,E*, and 4% *E,E*, and its purity was upgraded by HPLC to 94% *E,Z*, and 6% *E,E*. The material used in 1991 and 1992 was similarly purified to 95% *E,Z* from a separate but similar batch of crude material. After saponification of the purified acetate with KOH–methanol, the alcohol was used directly or oxidized to the aldehyde with CrO_3–pyridine–methylene chloride (Ratcliffe and Rodehorst, 1970).

Evaluation of Lures and Insect Traps. Lures for all traps in both the New York and Maryland studies were prepared by adding the pheromone components in hexane or methylene chloride to rubber serum stoppers (A. H. Thomas #1780 J07). GC examination of ethanol extracts from the stoppers showed no contamination with 1,2-dianilinoethane (*cf.* Steck *et al.*, 1979).

Experiments in New York in 1980 were carried out near Geneva and Ithaca. Trapping was done with Pherocon 1C traps in fields near hedgerows containing *P. serotina*. Traps were set out in a randomized complete block design, with a minimum of 10 m between treatments, and were sampled and rerandomized as indicated in the tables.

For the studies in Maryland, traps were placed along roadsides and in suburban yards near infested *P. serotina* trees in the Beltsville and Laurel areas. Treatments within a replicate were placed at least 10 m apart and replicates were separated by a distance of from several hundred meters to 10 km. Males were counted daily and each trap was then moved (rotated) to the next position in the row. Since some of the replicates were set out along heavily traveled roadsides, this trap rotation was considered more expeditious than rerandomization. Treatments were set out only in years of high populations, as indicated by defoliation of wild cherry trees and numbers of wandering stage larvae. Exact population levels were not determined, but should be comparable for the years presented.

In Maryland (1988 and 1989), both Pherocon 1C traps and water pan traps were used. The water pan traps (Figure 1) consisted of a removable trapping assembly and a retainer. The retainer was a 30-cm-diameter disposable aluminum pie plate nailed to the top of a wooden stake approximately 1 m tall. These were placed *ca.* 10 m apart along suburban roads or in yards. The removable assembly consisted of two facing pie plates, separated by a wooden dowel of sufficient length (*ca.* 12.5 cm) to give a separation at the edges of *ca.* 7.5 cm. This assembly was fastened inside the plate on the stake with spring clamps and contained a 1- to 2-cm layer of soapy water. The lure was held on a wire supported by the upper plate and approximately over the center of the lower plate. The soapy water was replaced as necessary.

For tests in Maryland in 1991, water pans were used to assess longevity of lures. Four replicates of a single treatment were set out for each of three consecutive trials. Traps were checked daily. Lures contained, in addition to

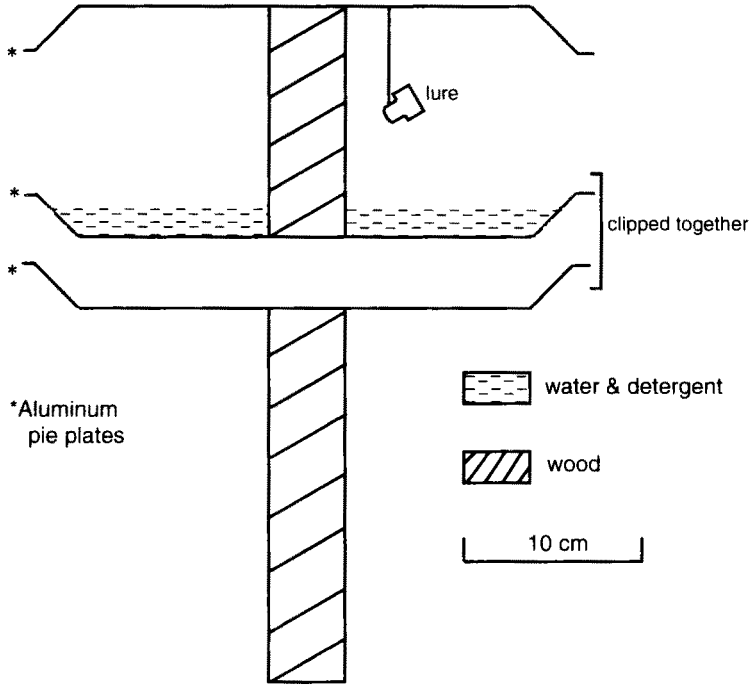

FIG. 1. Cross-sectional diagram of water pan trap. For further details, see text.

pheromone components, BHT and 2-hydroxy-4-methoxybenzophenone as antioxidant and photostabilizer, respectively (Ideses and Shani, 1988).

Field tests in Maryland in 1992 and 1994 were carried out with Pherocon 1C traps. The lures also contained antioxidant and photostabilizer as above.

Data from all tests were transformed $\sqrt{(X + 0.5)}$ and subjected to ANOVA. Data from 1980 tests in New York were analyzed using the Waller and Duncan BSD test under Minitab release 80.1 (Pennsylvania State University). Maryland data were analyzed within the SAS General Linear Models Procedure (SAS Institute, Raleigh, NC) with means separated by Duncan's multiple range test. Data for 1992 and 1994 were also analyzed with the Bonferroni *t*-test with equivalent results.

RESULTS AND DISCUSSION

Gas Chromatography and Electroantennogram Data. The crude extract from 30 female tips was injected onto a nonpolar OV-101 column at 150°C and

the effluent fractions collected as described above. EAG examination of these GC fractions showed activity at 4–5 and 6–7 min (2.0 mV and 1.4 mV, respectively, 1.0 mV at 5–6 min, all others were 0.8 mV or below). Similar analysis of collected airborne-material from 30 females showed activity at 4–5 min (4.8 mV) and 6–7 min (3.7 mV, all others 2.6 mV or below, average of three determinations on the same sample). Retention times of standards were 10:OH (2.5 min), 11:OH (3.9 min), 12:OH (6.0 min), 14:OH (15.3 min), 10:OAc (4.5 min), 11:OAc (7 min), and 12:OAc (11.05 min). The 4- to 5-min extract fraction from OV-101, injected onto a Hi-Eff column at 150°C, gave activity at 6–7 min (3.6 mV), 7–8 min (1.4 mV), and 8–9 min (2.6 mV, background 0.4–0.6 mV), as did the collected fraction from the airborne collection (2.0 mV, 7- to 8- and 8- to 9-min fractions at background of 0.4 mV). The crude extract fraction collected from OV-101 at 6–7 min, reinjected similarly onto the Hi-Eff column, gave activity at 14–16 min (1.4 mV); the airborne collection fraction reinjected gave activity at the same time (3.6 mV, 12–14 min at 1.8 mV, all other fractions at or below 1.4 mV). Hi-Eff retention times of standards were 11:OH (4.85 min), 12:OH (7.85 min), 14:OH (18.3 min), 11:OAc (4.6 min), 12:OAc (7.0 min), and 14:OAc (17.0 min). From these data and our earlier results (Hill and Roelofs, 1980), the pheromone seemed to consist of a mixture of a diunsaturated 12-carbon alcohol and aldehyde.

EAG Standards. Of a standard series of 12- and 14-carbon alcohols and acetates puffed across the male antenna, the greatest activity was elicited by the Z5 followed by the Z7 alcohol. This suggested, by analogy with similar cases (*e.g.*, Roelofs *et al.*, 1971; Kochansky *et al.*, 1975), that the pheromone was a 5,7-diunsaturated system comprising a mixture of alcohol and aldehyde. Diunsaturated standards (gifts of Warren Steck and Mel Chisholm, National Research Council, Saskatoon, Saskatchewan, and Robert Carney, Zoecon Corp., Palo Alto, California) showed the greatest activity from the *E*5,*Z*7-12:Ald among the aldehydes, and from *E*5,*Z*7-12:OH among the alcohols at a level of 100 ng on filter paper. Repeated runs at 100 ng and runs at a level of 10 ng gave similar results. Acetates were essentially inactive.

Evaluation of Lures. In the initial test in New York, the *EZ* aldehyde at a 1-mg level with 0.1 or 1 mg of *EZ* alcohol or 3 mg aldehyde alone were equally active (Table 1), but the aldehyde alone at 1 mg was less active than was the 1 mg + 1 mg treatment. Addition of 0.1 mg of *EZ* acetate to 1 mg of the aldehyde did not significantly reduce the number of males, but reduction occurred with an addition of 1 mg of acetate. Treatments containing other isomers were indistinguishable from blank traps. The second test covered the *EZ* aldehyde and mixtures with the alcohol at higher loadings. The aldehyde by itself at a 2-mg level was no more attractive than the blank trap, but the addition of *EZ* alcohol at levels of 0.2, 2, or 6 mg increased activity significantly.

TABLE 1. FIELD TRAPPING OF EASTERN TENT CATERPILLAR IN GENEVA AND ITHACA, NEW YORK, 1980

Treatment	Males/treatment[b]
Test 1 (June 26–July 10)[a]	
1 mg *E,Z* Ald + 1 mg *E,Z* OH	25.7 a
1 mg *E,Z* Ald + 0.1 mg *E,Z* OH	23.0 ab
3 mg *E,Z* Ald	17.7 abc
1 mg *E,Z* Ald	16.0 bc
1 mg *E,Z* Ald + 0.1 mg *E,Z* Ac	14.7 c
1 mg *E,Z* Ald + 1 mg *E,Z* Ac	7.0 d
1 mg *Z,E* Ald + 1 mg *E,Z* OH	1.3 e
1 mg *Z,E* Ald + 0.1 mg *E,Z* OH	0.7 e
1 mg *Z,E* Ald	0.7 e
1 mg *E,E* Ald	0.3 e
1 mg *Z,Z* Ald	0.3 e
Blank	1.0 e
	Total/treatment[d]
Test 2 (July 11–July 28)[c]	
2 mg *E,Z* Ald + 2 mg *E,Z* OH	55 a
2 mg *E,Z* Ald + 6 mg *E,Z* OH	48 a
2 mg *E,Z* Ald + 0.2 mg *E,Z* OH	43 a
2 mg *E,Z* Ald	2 b
Blank	0 b

[a] Three replicates, nine rerandomizations.
[b] Mean number of males trapped per treatment. Means followed by the same letter are not significantly different at $P = 0.05$ by the Waller and Duncan BSD test.
[c] Five replicates, six rerandomizations.
[d] Total males caught per treatment over the course of the test. Totals followed by the same letter are not significantly different at $P = 0.05$ by the Waller and Duncan BSD test.

The first field tests in Maryland used various designs in an attempt to optimize trap catch. Males of *M. americanum* are densely covered with hairlike scales, thus sticky traps frequently show only the scales left after a male escapes. In 1988, sticky traps were used in the initial series and low doses of the aldehyde and alcohol were compared with live females. Only two replicates were used because of the scarcity of females, which had to be replaced daily. Six males were caught by two traps with mixtures of aldehyde + alcohol (1 μg each) over seven days, 18 males were caught at the 10-μg level, and two females per trap caught 16. The second 1988 test compared water pan traps to sticky traps. Aldehyde and alcohol (100 μg each) in Pherocon 1C traps caught 16 males over 28 trap nights, while the same lure with water pan traps caught 24. The alcohol alone caught 3, and 1 mg of the alcohol + 600 μg of the aldehyde caught 28.

This represents an average of at most 1 male/trap/night (maximum catch was five males in a trap overnight). In neither of these studies were catches high enough for the differences to be significant.

The studies in Maryland in 1991 were intended to examine longevity of lures, but due to a pheromone shortage, tests were run only on a very small scale. Mixtures of aldehyde and alcohol (100 μg each, plus BHT and photostabilizer, four replicates) caught totals of 1, 6, 4, 0, and 0 males on successive nights. The lures were replaced with others at a level of 500 μg each with the same stabilizers, and caught 9, 4, 6, 0, 4, 0, and 0 males on successive nights. New 500-μg lures were put out and caught 3, 1, 3, 1, 0, and 0 males. Since these were not out at the same time, they cannot be directly compared, but it is apparent that even with the stabilizers, the lures' attractiveness decreased after a few days. This is hardly surprising, given the well-known susceptibility of multiply-unsaturated compounds to oxidation and isomerization.

For the first test of 1992 (Table 2), the aldehyde + alcohol (500 + 50 μg) treatment was more attractive than the mixture of the same components at 500 + 500 μg. At a level of 50 + 500 μg, attractancy of the mixture was no different from that of a blank trap. The second 1992 test involved closer ratios, but the differences between treatments in this test were not statistically significant.

In 1994, observations made over the previous years were used to design what we hoped would be a greatly improved test. Since Pherocon 1C traps were

TABLE 2. FIELD TRAPPING OF EASTERN TENT CATERPILLAR, BELTSVILE AND LAUREL, MARYLAND, 1992

Treatment	Total males[b]
Test 1 (June 12–June 15)[a]	
EZ Ald + *EZ* OH (500 μg + 50 μg)	26 a[c]
EZ Ald + *EZ* OH (500 μg + 500 μg)	15 b
EZ Ald + *EZ* OH (50 μg + 500 μg)	3 c
Blank trap	0 c
Test 2 (June 16–June 19)[a]	
EZ Ald + *EZ* OH (500 μg + 500 μg)	28[d]
EZ Ald + *EZ* OH (500 μg + 250 μg)	48[d]
EZ Ald + *EZ* OH (500 μg + 100 μg)	45[d]
EZ Ald (500 μg)	12[d]

[a] Four treatments, five replicates; sampled and rotated daily.
[b] Total males per treatment.
[c] Treatments followed by the same letter are not significantly different at $P = 0.05$ by Duncan's Multiple Range Test or by the Bonferroni t test.
[d] Total males per treatment. Differences were not significant at $P = 0.05$ by Duncan's multiple range test or by the Bonferroni t test.

TABLE 3. EASTERN TENT CATERPILLAR FIELD TRAPPING RESULTS, BELTSVILLE AND LAUREL, MARYLAND (JUNE 8–JUNE 16, 1994)[a]

Treatment	Males/trap/night[b]
EZ Ald + *EZ* OH (500 μg + 100 μg) (94% *EZ*)	19.09 a
EZ Ald + *EZ* OH (500 μg + 250 μg) (94% *EZ*)	16.41 a
EZ Ald + *EZ* OH (500 μg + 250 μg) (58% *EZ*)	8.59 b
Blank trap	0.0 c

[a] Four treatments, six replicates; sampled, rotated, and lures replaced daily. Traps had extra adhesive applied.

[b] Means followed by the same letter are not significantly different by Duncan's multiple range test or by the Bonferroni *t* test at $P = 0.05$. Total catches per treatment over the course of the test were 1118, 885, 464, and 0, respectively.

not sticky enough for this species, several millimeters of extra Tack Trap (Animal Repellents, Inc., Griffin, Georgia) were added to the trap bottoms. Lures were replaced daily and antioxidant and photostabilizer were added to help overcome the apparent rapid loss of activity. Trap bottoms were replaced as soon as they approached capacity. Greatly improved catches were finally obtained (Table 3). Mixtures of *EZ* aldehyde and alcohol (500 μg + 250 μg or 500 μg + 100 μg, respectively, were equally attractive) prepared from purified components were more attractive than the mixture (500 μg + 250 μg) prepared from the crude alcohol.

CONCLUSION

The best scheme found for trapping *M. americanum* was use of *E*5,*Z*7–12:Ald + *E*5,*Z*7–12:OH (purified on a $AgNO_3$ column) with a level of aldehyde at 0.5–1 mg and the level of alcohol lower by a factor of 2–5. Lures should be replaced daily in sticky traps with extra adhesive. The combination of these techniques enabled mean catches of 15–20 males/trap/day. Although mixtures of pheromone components synthesized from unchromatographed *EZ* acetate were attractive, they were less so than when the purified materials were used.

Since *M. disstria* uses the *ZE* isomer of the aldehyde and alcohol components, and *M. americanum* uses the *EZ isomer*, this provides a mechanism for reproductive isolation, in addition to the differences reported for the time of flight (Sample, 1992). The western tent caterpillar *M. californicum* uses the same major component as *M. americanum*, but since the two species are only marginally sympatric [distribution maps in Stehr and Cook (1968) and Myers (1993)], this should present only a limited problem, even assuming that all the

M. californicum subspecies use the same pheromone. The mechanism of isolation for these two species has apparently not been investigated, however.

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SEX PHERMONE COMPONENTS OF MULBERRY LOOPER, *Hemerophila atrilineata* BUTLER (LEPIDOPTERA: GEOMETRIDAE)

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Abstract—(6*Z*-9*S*, 10*R*)-Epoxy-octadecene (*SR*-**1**) and (3*Z*, 6*Z*-9*S*, 10*R*)-epoxy-octadecadiene (*SR*-**2**) are sex pheromone components of the mulberry looper (MBL), *Hemerophila atrilineata* Butler. Compounds extracted from female MBL pheromone glands were identified by coupled gas chromatographic–electroantennographic detection (GC-EAD) and GC–mass spectrometry. In field experiments in China, *SR*-**2**, *RS*-**2**, or both combined were hardly attractive, but *SR*-**2** in combination with *SR*-**1** attracted significant numbers of MBL males. Synergistic behavioral activity of *SR*-**1** plus *SR*-**2**, but not of corresponding antipode mixtures, indicates enantiospecificity of MBL pheromone communication. Because blends of racemic and enantiospecific (*SR*) **1** plus **2** were similarly attractive, racemic **1** plus **2** may have potential for mass trapping or confusion of MBL males in commercial mulberry plantations.

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INTRODUCTION

The mulberry looper (MBL), *Hemerophila atrilineata* Butler (Lepidoptera: Geometridae), is a widespread pest in plantations of mulberry trees, *Morus alba* (L.), throughout the provinces of Jiang Su, Zhe Jiang, Si Chuan, Guang Dong, and Liao Ning in China. Mulberry constitutes the food source for silkworm larvae, *Bombyx mori* L. (Lepidoptera: Bombycidae), raised for production of valuable silk. Sympatric MBL larvae of the spring generation eat buds of mulberry and subsequent generations defoliate trees. Reducing or destroying the food source for *B. mori*, MBL directly interferes with silk production, causing revenue losses to the silk industry. Because insecticides for MBL control would also affect highly sensitive silkworms, methods for MBL management are limited in number and scope. Use of the MBL-specific sex pheromone for mass trapping MBL males offered a potential control tactic. We report the identification of two sex pheromone components of MBL.

METHODS AND MATERIALS

Laboratory Analysis. MBL pupae were field collected in the Su Zhou area of the Jiang Su province and were sent to Simon Fraser University. Pupae were kept in filter paper-lined Petri dishes at a 14L : 10D photoperiod. Emergent moths were separated to avoid mating. One hour into the scotophase, pheromone glands of 1- to 2-day-old virgin females were removed and extracted in hexane for 5 min. Aliquots of one female equivalent of gland extract were subjected to gas chromatographic analysis (Hewlett Packard 5890A) with both flame ionization and electroantennographic detection (GC-EAD) (Arn et al., 1975; Struble and Arn, 1994) on two fused silica column (30 m × 0.25 or 0.32 mm ID) coated with DB-210 or DB-23 (J & W Scientific, Folsom, California). Coupled GC–mass spectrometric (MS) analyses of volatiles in gland extracts were carried out employing a Hewlett Packard 5985B fitted with the DB-210 column referred to above.

Synthetic chemicals, including (6*Z*-9*S*,10*R*)-epoxy-octadecene (*SR*-**1**), (6*Z*-9*R*,10*S*)-epoxy-octadecene (*RS*-**1**), (3*Z*,6*Z*-9*S*,10*R*)-epoxy-octadecadiene (*SR*-**2**) and (3*Z*,6*Z*-9*R*,10*S*)-epoxy-octadecadiene (*RS*-**2**), were synthesized as described by Miller et al. (1987, 1990) and were generously provided by the National Research Council, Saskatoon,Saskatchewan, Canada. Chemicals were >97% chemically pure and had enantiomeric excess (ee) of about 69%.

Field Experiments. Field experiments at Wujiang, China, were set up in randomized, complete blocks with traps and blocks at 30 to 40-m intervals. Traps consisted of an open, soapy water-filled bowl (25 cm diam.), with the pheromone lure suspended by wire in the trap center 2 cm above the water surface. Traps were hung from trees 1 m above the ground and baited with rubber septa (Sigma Chemical Co., St. Louis, Missouri) impregnated with Candidate pheromone components in HPLC-grade hexane. The first experiment (1993) tested *RS*-**2** (100 μg) and *SR*-**2** (100 μg) singly and in binary combination with *RS*-**1** (10 μg) or *SR*-**1** (10 μg). The second experiment (1994) tested *SR*-**2** (50 μg) and *RS*-**2** (50 μg) singly and in combination. Experiment 3 (1994) tested *SR*-**2** (50 μg) singly and in combination with either *SR*-**1** (5 μg), *RS*-**1** (5 μg), or both. Experiment 4 (1995) tested *SR*-**2** (50 μg) plus *SR*-**1** (5 μg) alone or in combination with either *RS*-**2** (50 μg), *RS*-**1** (5 μg), or both.

Statistical Analysis. Data were subjected to nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means (Student-Newman-Keuls test) (Zar, 1984; SAS/STAT User's Guide, 1988, release 6.03 edition, SAS Institute, Cary, North Carolina 27513).

RESULTS

GC-EAD analyses of female MBL pheromone gland extract revealed six compounds consistently eliciting antennal responses (Figure 1). GC-MS and retention index calculations of the major EAD-active compound (**2** in Figure 1) on several columns indicated a C_{18} diunsaturated epoxide. Subjecting 50 pg of standards of racemic 3, 4-, 6,7-, and 9,10-epoxy-dienes (C_{16}–C_{21}) to GC-EAD analyses revealed that 9,10-epoxy-dienes elicited the strongest antennal responses (Figure 2). (3*Z*, 6*Z*-*cis*-9,10)-Epoxy-octadecadiene had retention, mass spectrometric, and EAD characteristics highly comparable with female MBL-produced **2**. Because 3,4- and 6,7-epoxy-dienes had little EAD activity (Figure 2), and monoene epoxides elute just before diene epoxides on a DB-210 column, EAD-active compound **1** (Figure 1) was hypothesized to be (3*Z*- or 6*Z*-*cis*-9,10)-epoxy-octadecene. In GC-EAD analyses of 6,7- and 9,10-epoxy-monoenes (C_{17}–C_{20}), (6*Z*-*cis*-9,10)-epoxy-octadecene gave the strongest antennal response (Figure 3) and cochromatographed with female-produced **1** on DB-210 and DB-23 columns. Compounds **3**, **4** and **5**, **6** (Figure 1) had retention indices indicative of lower (**3**, **4**) and higher (**5**, **6**) homologs of **1** and **2**, but quantities in gland extracts did not suffice to confirm these structural assignments in spectrometric analyses.

In the first field experiment, *SR*-**2** in binary combination with *SR*-**1** was most attractive to MBL males (Figure 4), whereas mixtures of their antipodes were significantly less attractive. Singly *SR*-**2** or *RS*-**2** were as ineffective as

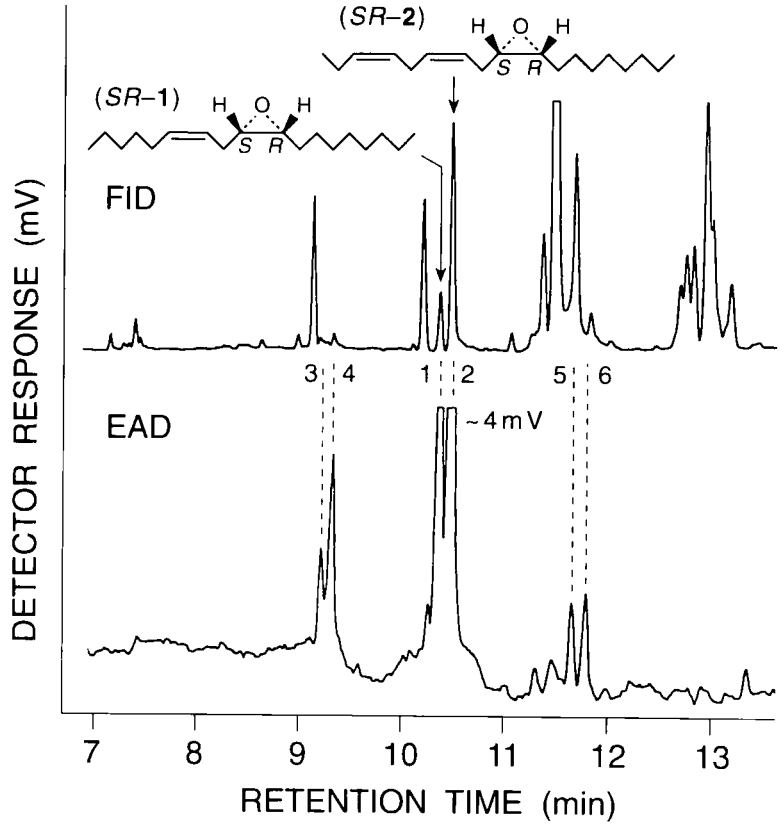

FIG. 1. Flame ionization detector (FID) and electroantennographic detector (EAD: male *H. atrilineata* antenna) responses to one female equivalent of female *H. atrilineata* pheromone gland extract. (6*Z*-9*S*,10*R*)-Epoxy-octadecene (*SR*-**1**) and (3*Z*,6*Z*-9*S*,10*R*)-epoxy-octadecadiene (*SR*-**2**) invariably elicited the strongest antennal responses. Chromatography: splitless injection; injector and FID detector temperature: 240°C; DB-210 column; temperature program: 100°C(1 min), 15°C/min to 180°C, 2°C/min to 220° C.

unbaited traps (experiment 1, Figure 4), and in combination (experiment 2) they failed to attract MBL males. *SR*-**2** was again unattractive in experiment 3, but in combination with either *SR*-**1**, *RS*-**1**, or both attracted a total of 10,6, and 12 MBL males, respectively. *SR*-**2** plus *SR*-**1** alone or in binary and ternary combination with *RS*-**2** and *RS*-**1** (experiment 4) were similarly effective, attracting a total of three to eight MBL males per treatment.

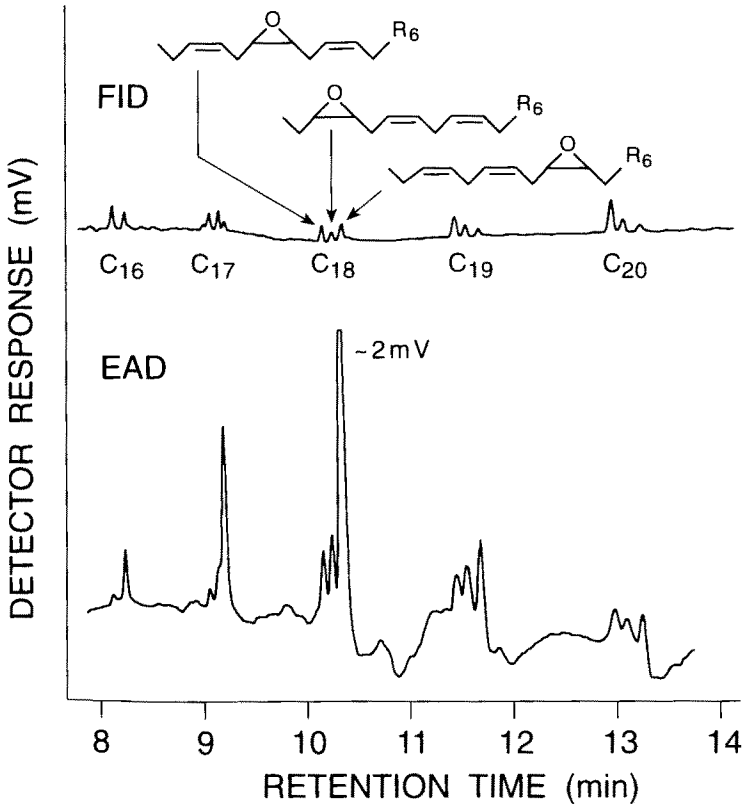

FIG. 2. Flame ionization detector (FID) and electroantennographic detector (EAD: male *H. atrilineata* antenna) responses to 50-pg standards of C_{16}–C_{20} epoxy-dienes. Chromatography as in Figure 1.

DISCUSSION

Evidence that (*Z*6-9*S*,10*R*)-epoxy-octadecene (*SR*-**1**) and (*Z*3,*Z*6-9*S*,10*R*)-epoxy-octadecadiene (*SR*-**2**) are sex pheromone components in female MBL include: (1) female MBL-produced and synthetic *SR*-**2** gave identical mass spectra, cochromatographed on several GC-columns, and elicited comparable antennal responses; (2) antennal responses to synthetic *SR*-**1** and female-produced **1** [second strongest EAD-active compound in pheromone extract (Figure 1)] had identical retention times on DB-23 and DB-210 columns; (3) synthetic *SR*-**1** and

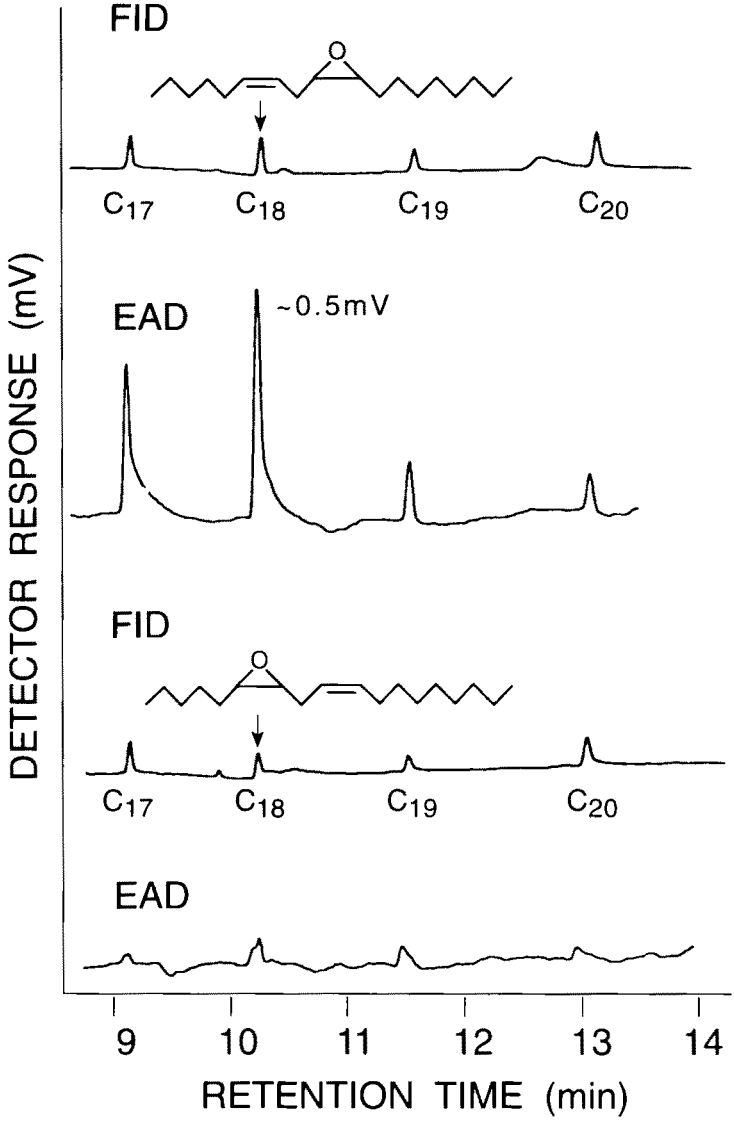

FIG. 3. Flame ionization detector (FID) and electroantennographic detector (EAD: male *H. atrilineata* antenna) responses to 50-pg standards of C_{17}–C_{20} monoene-epoxides. Chromatography as in Figure 1.

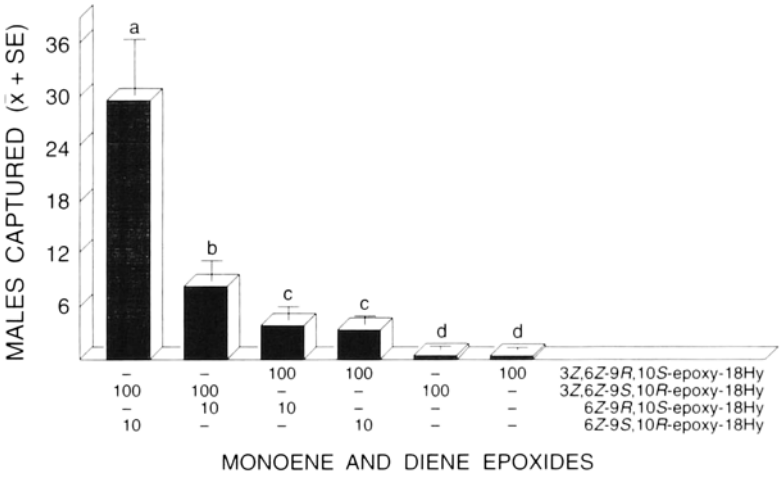

FIG. 4. Captures of *H. atrilineata* males in traps baited with (3*Z*,6*Z*-9*S*,10*R*)-epoxy-octadecadiene (100 μg) [3*Z*,6*Z*,-9*S*,10*R*-epoxy-18Hy (*SR*-**2**)], (3*Z*,6*Z*,-9*R*,10*S*)-epoxy-octadecadiene (100 μg) [3*Z*,6*Z*,-9*R*,10*S*-epoxy-18Hy (*RS*-**2**)], or either one of these epoxides in binary combination with (6*Z*-9*R*,10*S*)-epoxy-octadecene (10 μg) [6*Z*-9*R*,10*S*-epoxy-18Hy (*RS*,**1**)] or with (6*Z*-9*S*,10*R*-epoxy-octadecene (10 μg) [6*Z*-9*S*,10*R*-epoxy-18H6 (*SR*-**1**)] at the campus of Biotechnology College, Su Zhou University, China; June 9–12, 1993; eight replicates. Bats with the same superscript are not significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means (Student-Newman-Keuls test), $P < 0.05$.

SR-**2** rather than their antipodes elicited strong antennal responses; and (4) *SR*-**1** plus *SR*-**2** were highly attractive in field experiments (Figure 4).

Lack of attraction of MBL males to *SR*-**2** alone but strong attraction to *SR*-**2** plus *SR*-**1** indicates that pheromonal attraction in MBL resides with the presence of (at least) two pheromone components. EAD-active compunds **3**, **4** and **5**, **6** (Figure 1) are likely lower and higher homologs of **1** and **2**, but ultra low MBL population densities in 1994–1995, seriously affecting experiments 2-4, did not allow exploration of their potential behavioral activity. Attractiveness of *SR*-**1** plus *SR*-**2**, but not of corresponding antipode mixtures, indicates enantiospecificity of pheromone communication in MBL. Because blends of *SR*-**1** plus *SR*-**2** or their racemates were similarly attractive (experiment 4), antipodes of MBL pheromone components are either behaviorally benign or not perceived by MBL males.

Enantioselective pheromone production by female MBL has not yet been demonstrated unequivocally, but the technology required for such analyses has recently been developed. Employing specific gas chromatographic conditions

and a tailor-made GC column (König et al., 1992; Pietruszkae et al., 1992), Szöcs et al. (1993) succeeded in separating optical isomers of (3*Z*,9*Z*-*cis*-6,7)-epoxy-nonadecadiene, an epoxy-diene with an even longer chain length than MBL pheromone components.

Epoxides *SR*-**1** and *SR*-**2** are essential for attraction of MBL males and have not previously been reported as sex pheromone components in the Lepidoptera. Multiple phermone component blends in other geometrid moths comprise optical isomers of epoxy-dienes (Millar et al., 1990), epoxy-dienes plus triene hydrocarbons (Millar et al., 1987; Tóth et al., 1992), triene plus diene hydrocarbons (Millar et al., 1990), mono- and dimethyl hydrocarbons (Gries et al., 1991, 1993, 1994), and optical isomers of dimethylated hydrocarbons (King et al., 1995).

Similar attractiveness of enantiomerically enriched *SR*-**1** plus *SR*-**2** and racemic **1** plus **2** (experiment 4) suggests that antipodes of pheromone components do not interfere with the attraction of MBL males. Should racemic **1** plus **2** be as attractive as virgin MBL females, this mixture should be evaluated for mass trapping MBL males or for their disorientation in pheromone-based mating disruption.

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AGGREGATION PHEROMONE SYSTEM OF NYMPHAL GREGARIOUS DESERT LOCUST, *Schistocerca gregaria* (FORSKÅL)

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Abstract—Hexanal, octanal, nonanal, decanal, hexanoic acid, octanoic acid, nonanoic acid, and decanoic acid were identified in the volatile emissions from fifth instars of *Schistocerca gregaria* by coupled gas chromatography-electroantennography (GC-EAD) detection and coupled GC-mass spectrometry (MS). In laboratory assays, synthetic blends of the eight compounds, with acids and aldehydes tested separately, evoked strong aggregation responses from fifth instars, similar to responses evoked by a crude volatile extract of the nymphs. A nymphal fecal volatile phenolic blend of guaiacol and phenol, which we have previously shown to evoke an aggregative response from different stages of the insect, significantly synergized the activity of the blend of eight compounds, suggesting mutually augmentative roles for nymphal and fecal volatiles in evoking aggregation. These results indicate that the aggregation behavior of gregarious second to fifth instars is modulated by three sets of pheromonal compounds: aldehydes and acids emitted by the nymphs themselves and phenols associated with their feces.

Key Words—*Schistocerca gregaria*, locusts, aggregation pheromone, hexanal, octanal, nonanal, decanal, hexanoic acid, octanoic acid, nonanoic acid, decanoic acid, guaiacol, phenol.

INTRODUCTION

Aggregation behavior in the gregarious desert locust *Schistocerca gregaria* is mediated by a complex pheromone system released in the volatile emissions of

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the different locust stages, sexes, and their feces (Obeng-Ofori et al. 1993, 1994a,b). The adults aggregate in response to a blend containing phenylacetonitrile, benzaldehyde, guaiacol, and phenol associated with older males (Torto et al., 1994). In the absence of older males, young adults aggregate in response to guaiacol and phenol present in their fecal volatiles and those of nymphal stages with which they associate during fledging (Obeng-Ofori et al., 1994b). In the second, third, fourth, and fifth instars we demonstrated that these nymphal stages respond to one another's volatiles and that no sexual differentiation occurs in the production of pheromone (Obeng-Ofori et al., 1993, 1994a).

We have expanded on these earlier findings by investigating the nymphal-produced pheromone system of *S. gregaria*. We chose as our experimental insects the fifth instars, and herein present the isolation, identification, and behavioral activity of its aggregation pheromone.

METHODS AND MATERIALS

Volatile Collection. Fifty fifth-instar nymphs (three to five days after molt) of mixed sexes were obtained from the ICIPE insectary (Torto et al., 1994), placed in a 5-liter three-necked, round-bottomed flask, and aerated (Obeng-Ofori et al., 1993). A charcoal-filtered airstream (106 ml/min) was drawn over the locusts by a vacuum pump (Cole-Parmer air-cadet) for 15 hr in a room maintained at 30 $\pm$ 2°C. Volatiles were collected on charcoal (120 mg) traps in Pyrex glass tubes (Torto et al., 1994) immersed in an ice bucket. The volatiles were eluted from the charcoal traps with HPLC-grade dichloromethane (5 ml, Aldrich Ltd.), dried over anhydrous Na_2SO_4, and stored in the freezer at -15°C until used.

Analyses of Volatiles. Volatiles were concentrated under a stream of nitrogen at 0°C to 100 μl. Eight microliters were analyzed by gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975) using conditions previously described on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with a FID detector and a Syntech EAD system (Torto et al., 1994). The GC oven was fitted with a HP capillary column (Carbowax 20 M, 50 m $\times$ 0.2 mm ID $\times$ 0.2 μm) and temperature programmed as follows: 60°C (10 min), 5°C/min to 180°C, held for 5 min, and raised at 10°C/min to 220°C. FID and EAD signals were monitored synchronously using a program on a GC/EAD interface card (Syntech) installed in a PC (Harvard Professional Computer, American Megatrends Inc.). The signals were recorded at a fixed speed of 9.2 cm/min and stored as pages of 128 sec (2 min 8 sec) duration. One complete run using the GC-EAD conditions previously described (Torto et al., 1994) generates 25 pages. In addition, the FID signal was plotted on a HP 3396 Series II integrator at 0.5 cm/min. GC-MS analyses were carried out on a VG Masslab 12-250 mass spectrometer (EI, 70 eV) coupled to a HP 5790 gas chromatograph.

Behavioral Assays. A modification of the olfactometer bioassay described previously (Obeng-Ofori et al., 1993) was used to examine aggregation responses by fifth instars and older adults to natural and synthetic pheromones. The bioassay arena was fitted with three pieces of removable wire mesh, one split into two halves, with each half fitted at the bottom of the chamber on the perforated aluminum metal plate, and another fitted in the middle of the chamber while the other piece of wire mesh covered the top (Figure 1). Ten filter paper discs (each 16 mm diameter) were distributed on each of the pieces of wire mesh

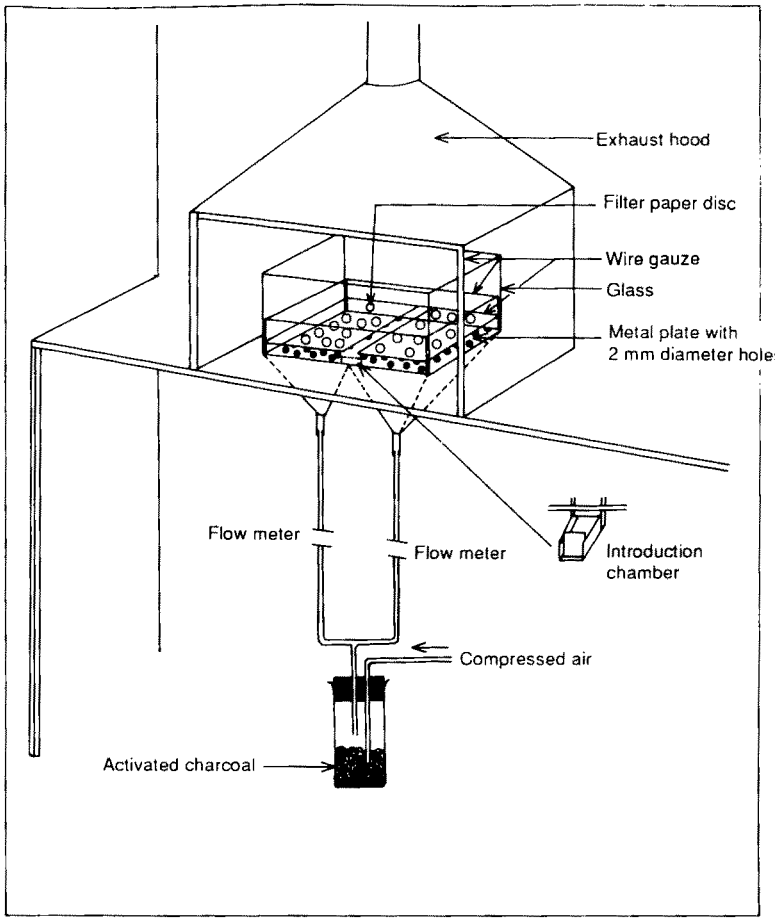

FIG. 1. Diagram of modified single-chamber choice olfactometer bioassay and layout of experimental arena.

placed at the bottom of the chamber. Pheromone extracts in paraffin oil were applied to one set of discs and the control (paraffin oil) on the other set. This modification was necessary to facilitate easy flow of test substances, which are of low volatility, into the airstream of the bioassay arena. Charcoal-filtered air, split into two flow-metered streams, was directly passed into the square pyramidal aluminum funnel attached to the base of the chamber. To avoid accumulation and mixing of the eluate in the assay chamber, the effluent from the olfactometer was extracted into a hood by a fan through a duct.

Bioassays provided a choice of an odor-free control air column and an air column treated with: (1) a volatile collection of fifth instars (crude extract) corresponding to 300, 150, 75, and 37.5 locust hours (LH) (1 LH = volatiles emitted by one locust for 1 hr) (Torto et al., 1994); (2) a 4:9:20:20:14:50:100:16 blend of synthetic hexanal, octanal, nonanal, decanal, hexanoic acid, octanoic acid, nonanoic acid, and decanoic acid (the ratio represents the relative composition of a volatile collection from a single sample of a population of fifth instars at the four doses described in (1); (3) a blend of the aldehydes in relative concentrations present in (2); (4) a blend of the acids also in relative concentrations present in (2); (5) the individual aldehydes as in (3); (6) the individual acids as in (4); (7) preparation as in (2) containing 5 ng each of guaiacol and phenol (nymphal fecal volatile components) (Obeng-Ofori, et al., 1994b); and (8) a blend containing 5 ng each of guaiacol and phenol. Control and treated sides were systematically switched throughout the study to minimize bias.

Sets of five locusts of mixed sexes were bioassayed and were released on the wire mesh which fitted in the middle part of the chamber, and after 10 min the number of insects in each section of the bioassay arena was counted. Uncommitted insects found in the middle part of the arena were treated as nonresponders. All tests were replicated 10 times, and locusts were used only once and discarded. The aggregation index was calculated as $100(T - C)/N$ where T is the number of locusts found in the treated compartment, C is the number of locusts in the control compartment, and N is the total number of locusts tested. If this calculation resulted in a negative number, the aggregation index was assumed to be zero. Aggregation responses were transformed into arcsine and submitted to analysis of variance to compare the effects of natural and synthetic pheromone on fifth-instar aggregation. Mean aggregation responses were compared using the LSD test ($\alpha = 0.05$).

RESULTS

Analyses of Volatiles. EAG-active peaks **1–8** in the crude nymphal volatile extract were identified as hexanal, octanal, nonanal, decanal, hexanoic acid,

TABLE 1. AGGREGATION RESPONSES OF CROWDED *S. gregaria* TO FIFTH INSTAR VOLATILES IN OLFACTOMETER

	Percent aggregation index (± SE)[a]									
	Crude volatiles (dose in LH)				Synthetic blend (dose in LH)				Overall	
Insect stage	37.5	75	150	300	37.5	75	150	300	Crude	Synthetic
Fifth-instar nymph	20(7)a	34(8)a	60(10)a	79(9)a	36(7)a	50(9)a	58(10)a	74(6)a	46(5)a	54(4)a
Older adults	4(3)b	2(2)b	0(0)b	2(2)b	4(3)b	6(3)b	16(8)b	20(9)b	2(1)b	11(3)b

[a]Means in a column followed by different letters are significantly different at $P < 0.05$.

octanoic acid, nonanoic acid, and decanoic acid, respectively, by GC-MS (Figure 2). Combined GC-EAD recordings using authentic samples of these compounds on Carbowax and methyl silicone columns confirmed the retention times and EAG activities observed with the natural volatile extract.

Six components with weak activity were identified after analyses of volatile collections from a large number of nymphs (240) as undecanal, dodecanal, pentanoic acid, heptanoic acid, undecanoic acid, and dodecanoic acid. Heptanal,

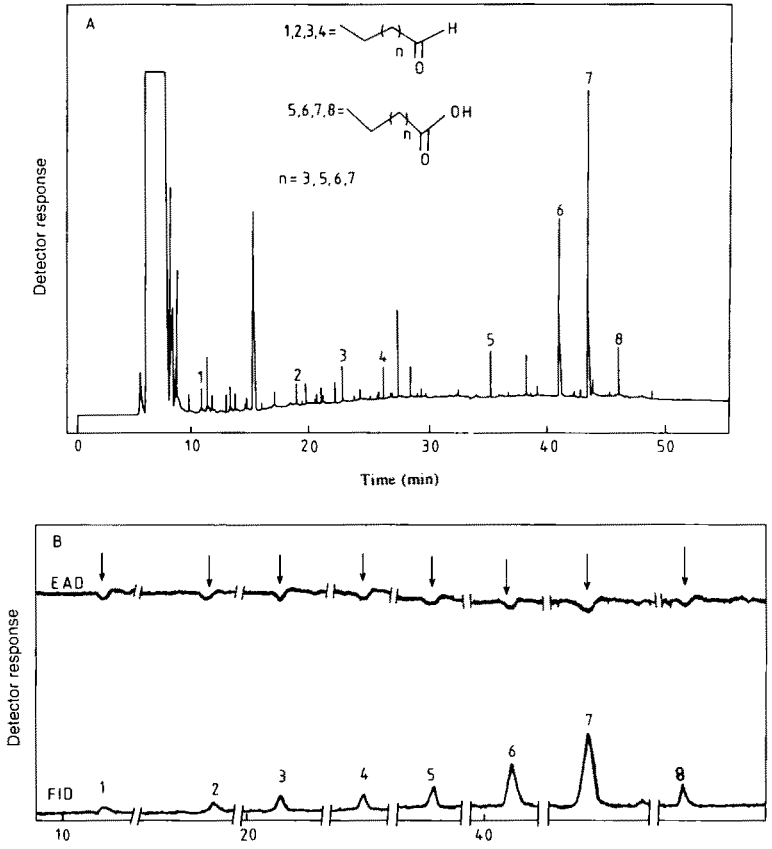

FIG. 2. (A) Gas chromatogram of volatiles collected from fifth-instar nymphs of *S. gregaria* injected onto a 50-m Carbowax 20 M capillary column, and (B) representative reconstructed computer GC-EAD recordings of female *S. gregaria* antennae, responding to compounds **1–8** in the crude nymphal volatile extract.

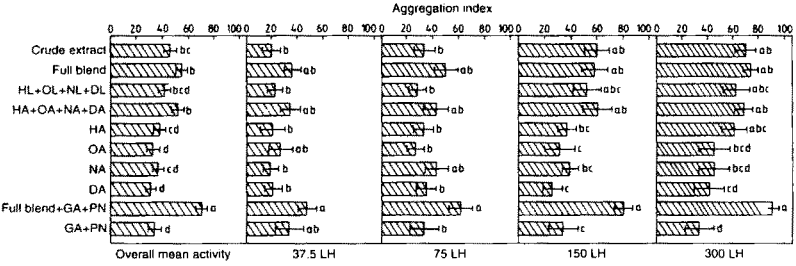

FIG. 3. Dose-response relationships for EAG-active components from fifth-instar volatiles at natural relative concentrations causing aggregation of the nymphs. Means at a particular dose followed by the same letter are not significantly different ($P < 0.05$) by LSD test (percentage transformed to arcsine before analysis). HL = hexanal, OL = octanal, NL = nonanal, DL = decanal, HA = hexanoic acid, OA = octanoic acid, NA = nonanoic acid, DA = decanoic acid, GA = guaiacol, PN = phenol. Full blend = HL + OL + NL + DL + HA + OA + NA + DA.

anisole, benzaldehyde, acetophenone, veratrole, guaiacol, and phenol were also detected in trace amounts.

Behavioral Assays. Responses by fifth instars to the pheromone treatments were dose-dependent (Table 1 Figure 3). Each of the blends consisting of the four acids and four aldehydes evoked strong aggregation responses from nymphs at all doses, similar to the crude extract and the full synthetic blend of the eight compounds. None of the individual aldehydes showed significant activity, but individual acids were moderately active. Addition of guaiacol and phenol to the full blend significantly enhanced aggregation responses in nymphs, particularly at lower doses compared to the crude extract. Overall, the synthetic blend of the 10 compounds evoked significantly stronger aggregation responses from nymphs than the crude extract or the different synthetic blends.

DISCUSSION

Our previous studies focused on the characterization of the aggregation pheromone system of adult gregarious desert locusts (Torto et al., 1994, Obeng-Ofori et al., 1994b). In the present study the volatiles produced by fifth-instars were examined. Eight electrophysiologically active compounds were identified consisting of hexanal, octanal, nonanal, decanal, hexanoic acid, octanoic acid, nonanoic acid, and decanoic acid. Fifth instars responded strongly to the full synthetic blend of the eight compounds, but the blend evoked no significant aggregation response from older adults Table 1, confirming our previous results

that demonstrated stage differentiation in the pheromone systems of gregarious *S. gregaria* (Obeng-Ofori et al., 1993). Individual aldehydes showed no significant activity. Earlier, we had shown that fifth instars aggregated in response to their fecal volatile components guaiacol and phenol (Obeng-Ofori et al., 1994b). Although only trace amounts of these phenols are present in volatile collections of the nymphs themselves, gregarious nymphs are likely to encounter relatively larger concentrations from their fecal droppings (Obeng-Ofori et al., 1994b). Addition of guaiacol and phenol to the full blend significantly enhanced aggregation responses in fifth instars. Thus, the aggregation behavior of nymphal desert locusts appears to be modulated by three sets of pheromonal compounds: short-chain aldehydes and acids produced by gregarious nymphs and guaiacol and phenol associated with their fecal volatiles, which act synergistically. Whether these compounds contribute to different behavioral elements that may be involved in the overall aggregation or have different primary roles in the process remains to be elucidated. The present bioassay was designed to measure gross aggregative effects of candidate materials, and a more detailed study involving the effects of different pheromonal components and blends on different elements of aggregation needs to be undertaken.

Some of the aldehydes and acids detected in the volatiles of the nymphs have been reported as plant constituents (Visser, 1986). The nymphs may synthesize the compounds *de novo* or obtain them from plants. Significantly, solitary nymphs reared on the same food as their gregarious counterparts emit trace amounts of these compounds, but they are not always detectable in their volatile emissions. Several different arthropods produce similar aldehydes and acids in their pheromone glands or volatile emissions, e.g., nonanal, produced in the male pheromone gland of *Galleria mellonella* (L.) (Romel et al., 1992); nonanoic acid, an aggregation–attachment pheromone of *Amblyomma variegatum* (Pavis and Barre, 1993); hexanal, a component in the pheromonal emissions of calling male Mediterranean fruit flies (Flath et al., 1993); and with its carboxylic acid derivative, hexanoic acid, as components of the alarm pheromone system of the leaf-footed bug *Leptoglossus zonatus* (Leal et al., 1994).

In summary, whereas the aggregation behavior of adult gregarious desert locusts is mediated by aromatic derivatives (Torto et al., 1994), aggregation of the second to fifth instars is mediated by straight-chain aliphatic aldehydes and acids that are synergized by fecal phenols. We are currently investigating the semiochemicals associated with aggregation of first-instar gregarious nymphs, which produce no significant amounts of the volatile aggregative signal components identified in gregarious fifth instars.

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SOME CHEMICAL BASES FOR GYPSY MOTH, *Lymantria dispar*, LARVAL REJECTION OF GREEN ASH, *Fraxinus pennsylvanica*, FOLIAGE AS FOOD

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Abstract—Green ash is one of the few tree species rejected as food by larvae of the generalist gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae). Such rejection is based especially on chemicals present in green ash foliage. The gypsy moth larval feeding-inhibitory activity is contained in the ethyl acetate extractables of green ash foliage. Three representative column-chromatographed fractions of the extractables contained antifeedant activity. Individual fractions showed weaker antifeedant activity compared to the total ethyl acetate extractables. Acid hydrolysis of the extractables destroyed antifeedant activity and yielded feeding stimulant activity at higher concentrations. The aqueous extractables were not feeding-inhibitory. Compounds in the green ash extractables were separated by TLC, HPLC, CC, and GC.

Key Words—Gypsy moth, green ash, feeding inhibition, *Lymantria dispar*, Lepidoptera, Lymantriidae, *Fraxinus pennsylvanica*, Oleacae.

INTRODUCTION

Herbivores are frequently exposed naturally to a wide variety of dissimilar plants. Such a situation makes nonhost rejection and host-plant selection major herbi-

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vore decisions. The unsuitability of nonhosts as food for herbivores is ultimately determined by overall plant defensive strategy, which may involve anatomical, morphological, and/or chemical parameters (Norris and Kogan, 1980). A comprehensive strategy better enables the successful survivor plant species to direct its defenses at the entirety of environmental stresses, whether biological, physical, and/or chemical, and not just at some specific stress, e.g., an herbivorous insect or microbial pathogen (Norris, 1988). Although a nonhost plant decision by most insects is affected by the plant's overall defensive capabilities, repellent and/or deterrent chemicals may play a major role. Feeding-deterrent compounds contribute significantly to the inability of herbivorous insects to use certain plants as hosts (Fraenkel, 1959; Jermy, 1966; Gilbert et al., 1967; Whittaker and Feeny, 1971; Norris, 1990, 1994; Burden and Norris, 1992) and may especially influence the feeding behavior of polyphagous insects (Meisner and Skatulla, 1975; El-Naggar et al., 1980; Doskotch et al., 1980; El-Naggar and Doskotch, 1980; Schultz and Baldwin, 1982; Miller and Feeny, 1983; Barbosa and Krischik, 1987; Bowers and Puttick, 1988, 1989). The polyphagous gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae), rejects foliage of the green ash tree, *Fraxinus pennsylvanica* Marsh (family Oleaceae), as a host (Mosher, 1915; Mauffette and Lechowicz, 1983; Odell et al., 1985; Lechowicz and Mauffette, 1986). Chemicals in green ash play a major role in this nonhost rejection.

The present paper is a contribution to a larger effort to define the continuum of chemical defenses in green ash against environmental stresses, which makes this tree a nonhost to the gypsy moth. Our earlier paper (Markovic et al., 1996) addressed locomotory responses of gypsy moth larvae to volatile chemicals emanating from green ash foliage. The present effort addresses the effects of ash-foliage chemistry on larval feeding.

METHODS AND MATERIALS

Insects

Gypsy moth egg masses were obtained from the Otis Air Force Base rearing facility in Massachusetts. If egg masses were not used immediately upon arrival, they were stored at +10°C in darkness. In preparation for hatch, egg masses were surface sterilized for 5 min in 0.1% sodium hypochlorite solution. After sterlization, egg masses were rinsed for 10 min with distilled water and dried.

Rearing was conducted on a wheat germ artificial diet in incubators at 25 ± 1°C with a 15 L:9 D photoperiod. Sterilized individual egg masses were placed aseptically into individual plastic dishes (4 × 15.5 cm) along with a piece of diet (10 × 1.0 × 1.5 cm). Sterile technique was employed throughout the study.

Plants

Leaves for the studies were collected on the University of Wisconsin campus from five green ash (*Fraxinus pennsylvanica* Marsh) trees that had not been recently exposed to aerial and/or other kinds of pesticide application.

Moisture Determination

The fresh weight of *F. pennsylvanica* leaves was determined immediately after sampling. Leaves were put into preweighed weighing bottles, dried for 24 hr at 110°C, cooled in a desiccator, and reweighed in the resealed bottle. Dry weight of the leaves was determined, and the percent of moisture per leaf calculated.

Chemical Analyses

Extraction Procedure. Green ash leaves (100 g) were randomly taken from each tree, placed immediately into a screw-cap glass jar containing 800 ml of 80% methanol (MeOH), and stored in darkness at −20°C until chemical analysis. All individual leaflets were removed from each compound ash leaf per jar, and the resultant denuded leaf petioles were cut into 0.5-cm-long sections. The petiole sections and individual leaflets per jar were combined in 800 ml of 80% MeOH for storage. For processing of samples, we added 100 ml of 100% MeOH and homogenized for 15 min at 50% maximum speed in an Omni-mixer (Ivan Sorvall, Inc., Norwalk, Connecticut). The resultant homogenate was filtered twice through Whatman No. 1 paper under vacuum. The filtrate was reduced to about 15 ml by rotoevaporation at 42 ± 2°C under vacuum. The concentrated MeOH extractables resulting from homogenization of 100 g (fresh weight) of leaves were partitioned in a separatory funnel (3 × 1000 ml of ethyl acetate). Ethyl acetate (EtOAc) fractions were rotoevaporated to dryness and redissolved in an excess of EtOAc (Markovic et al., 1993). Each sample was then placed in a preweighed screw-cap vial, dried under a stream of nitrogen, and weighed. Samples in screw-capped vials were wrapped in aluminum foil and stored at −20°C in darkness.

The remaining aqueous extractables after EtOAc partitioning of MeOH extractables were transferred into a preweighed screw-cap vial, lyophilized for 36–48 hr and the dry weight was determined. Vials with dry aqueous extractables were also stored at −20°C in darkness for later use.

Acid Hydrolysis of Green Ash Extractables. Acid hydrolysis to cleave glycosidic bonds (i.e., to separate algycones from sugars in glycosides) was performed on a 50-mg subsample of the dried EtOAc extractables from 100 g of leaves. This subsample was hydrolyzed 1.5 hr in 60 ml of 2 M hydrochloric acid in a water bath at 98 ± 2°C (Harborne, 1984). The resultant hydrolyzed

subsample was cooled, filtered through Whatman No. 2 paper, and then extracted with 35 ml of chloroform ($CHCl_3$) under vacuum. Extracting hydrolyzed material in ethyl ether (EtO) instead of $CHCl_3$ resolved fewer peaks when applied on the TLC plate. Finally, $CHCl_3$ extractables of each subsample were dried under a nitrogen stream, and their dry weights were determined.

Acid hydrolysis of aqueous extractables was performed in the same way as with EtOAc extractables except that hydrolyzed material was redissolved in EtO.

Fractionation of Green Ash EtOAc Extractables. Activated silica gel (50- to 200-μm particle size) was poured in a slurry of $CHCl_3$–MeOH (9:1) into two glass columns (16-ml and 400-ml volume). Prior to application of the plant material, each column was rinsed with six 25-ml portions of $CHCl_3$–MeOH (9:1). With the 16-ml column, 300 mg EtOAc extractables were applied and six 25-ml fractions were collected in $CHCl_3$–MeOH (9:1) (LaLonde et al., 1976). Fractions 5 and 6 were combined (6.9 mg of dried material) as fraction A, and used for bioassay. We applied 4 g of EtOAc extractables to the 400-ml column and eluted fractions using the following sequential solvent regime: 550, 100, 300, 250, and 250 ml of $CHCl_3$–MeOH (9:1); 150 ml of $CHCl_3$–MeOH (1:1); 200 and 300 ml of $CHCl_3$–MeOH (1:9); and next one 300- and five 200-ml quantities of $CHCl_3$–MeOH (9:1). The last two 200-ml fractions were combined (4.1 mg of dried material) as fraction B and used for bioassay. The preceding elution sequence on the 400-ml column was followed by four 200-ml fractions of 60% aqueous MeOH. The last of the four 200-ml fractions (3.7 mg of dried material) (fraction C) was bioassayed. Eluted fractions were filtered through Whatman No. 2 paper and dried by rotoevaporation or under a stream of nitrogen. Each fraction was then redissolved in an excess of 100% MeOH, passed through a Gelman Nylaflo 0.20-μm filter, and the dry weight determined for bioassay. The chemical profile of each fraction was monitored by HPLC and GC.

Thin-Layer Chromatographic (TLC) Analyses. Each 20 μl of a 10 mg/ml (w/v) sample was vortexed for 6 sec before being spotted with a glass capillary tube on a silica gel TLC plate (20 × 20 cm, 250 μm thick, 5- to 17-μm particle size, and a 254-nm fluorescent indicator) (Sigma Chemicals, St Louis, Missouri). Plates were then developed in a solvent mixture of chloroform–acetone–acetic acid (90:10:1) in a closed glass tank saturated with solvent vapors. The solvent was run up 65% of the plate height, and the plate was allowed to air dry at room temperature. Resolved bands were first detected under 254-nm UV light (Mineral Light Lamp, UVSL-25, Ultra Violet Products, San Gabriel, California). Resolved and dried TLC plates were next examined for resolved compounds with at TLC scanner under UV light (TLC SCAN II, CAMAG Scientific Inc., Wilmington, North Carolina 28405) combined with a CATS3 computing-integrator software program.

High-Performance Liquid Chromatographic (HPLC) Analyses. A 2.5- to 5.0-mg aliquot of each sample was dissolved in 1-ml HPLC-grade MeOH (Aldrich Chemical Company), vortexed for 1 min, and then passed through a Gelman Nylaflo 0.20-μm filter. Just before the injection into the HPLC, each sample was vortexed for 1 min, and a 20-μl volume injected into a Beckman Ultrasphere ODS 5-μ column (4.6 mm $\times$ 25 cm). The eluting solvent consisted of 2% acetic acid–acetonitrile (90% : 10%) for 5 min, a linear gradient to 100% acetonitrile in 25 min, and then back to the starting ratio in 3 min. There were three replicate injections per sample. The HPLC system was controlled by an Altex 422 programming microprocessor and two Altex 100A pumps. The UV detector (Kratos) was set at 254-nm absorbance. Data were integrated and peak areas recorded through a Spectra Physics 4400 computing integrator.

Capillary Gas Chromatography (GC) Analyses. The EtOAc extractables from green ash leaves, and individual fractions therefrom, were analyzed by gas chromatography using a Shimadzu GC-14A GC with a DB-17 capillary column (0.25 mm $\times$ 30 m, 0.25-μm film thickness, J&W Scientific, Folsom, California 95630) and a flame ionization detector (FID), or with a DB-1 capillary column (0.25 mm $\times$ 30 m, 0.25-μm film thickness; J & W) mated via a heated transfer line to a Finnigan MAT ITD 800 series mass spectrometer. The GC was programmed from 40°C for 1 min to 220°C, at 5°C/min. The injector temperature was 220°C and the detector, 265°C. Aliquots of 1–2 μl of the extracted sample were injected in the splitless mode. Retention time, area, and ratio of each resolved peak area to the total resolved peak area were calculated and recorded by a Shimadzu C-R3A Chromatopac computing integrator.

Feeding Bioassays

General Considerations. For feeding bioassays neonates were placed on artificial diet, and the larvae were reared until they reached the third instar. Third instars, 24- to 36 hr old, were removed from other larvae of a given egg mass and placed in groups of 20, with the result that each individual was from a different egg mass. They were then placed in a plastic dish (4.5 $\times$ 11.5 cm) containing a diet cube. Experimental larvae were uniformly starved by removing the cube of diet 4.5–5 hr prior to the initiation of the experiment. Transferring larvae from artificial diet to fresh leaf discs does not significantly disrupt their feeding (Hajek, 1989). An experiment was terminated when at least 50% of the area of a leaf disc in an arena was removed, or when 28 hr had elapsed.

Preference Between Red Oak and Green Ash Foliage. The nonhost/host status of *F. pennsylvanica* foliage regarding *L. dispar* was determined using a preference test. Twenty-four- to 36-hr-old third instars were used in feeding bioassays. Unless otherwise specified, 15 replicates per treatment were used with two larvae from a given egg mass constituting a replicate. Larvae were

provided with green ash and/or red oak leaf discs cut with a No. 8 (1.5-cm-diam) cork borer. The feeding arena consisted of a plastic Petri dish (1.3 × 9 cm) with a 2- to 3-mm-thick layer of paraffin on the bottom of the dish. One 9-cm-diam. No. 1 filter paper was attached to the inside surface of the lid of each arena and another on the paraffin bottom. The top paper was moistened as necessary to maintain the turgidity of the leaf discs. Two 1.2-cm-long insect pins secured each leaf disc to the paper-covered paraffin bottom of the arena.

For the two-choice assay, insects were offered a comparable leaf disc from green ash (treatment) versus red oak (control). Each leaf disc was placed about 1.5 cm from the edge of the arena, leaving about 1.5 cm centrally between the two discs in the arena setup. Two larvae were introduced centrally into the feeding arena.

In the no-choice assay, a single larva was offered either a green ash or a red oak leaf disk. An adjacent treatment- or control-containing arena was considered as the other member in this paired, but no-choice, assay.

Dose–Response Test with Nonhydrolyzed Extractables from Green Ash Foliage. A dose–response study determined the biologically active concentration range for the EtOAc extractables from green ash foliage. The following concentrations were employed: 0.025, 0.05, 0.10, 0.25, 0.5, 1.0, 2.0, 3.0, 5.0, 7.5, 10.0, 15.0 mg/ml (w/v) in 100% MeOH. Two oak (host) leaf discs were presented per assay. Forty microliters of an appropriate concentration of green ash extractables were presented as the treatment on the upper surface on one of the two leaf discs. An equal amount of 100% MeOH was presented as the solvent control on the upper surface of the other leaf disc. Each extractables-containing vial was vortexed 1 min before a sample was removed and applied on a leaf disc. Two third instars were placed centrally between the treated and control discs to initiate the assay.

A second dose–response study determined the presence or absence of biologically active components in the residual aqueous extractables from green ash foliage, and the range of active concentrations, if activity was detected. The following concentrations were employed: 0.1, 0.5, 2.0, 5.0 and 10.0 mg/ml (w/v) in 80% MeOH. The feeding bioassays comparing the activity of aqueous extractables versus the 80% MeOH control were conducted as described for the dose–response tests with EtOAc extractables.

Dose–Response Test with Hydrolyzed Extractables. The following concentrations were employed for the feeding bioassay: 0.10, 0.5, 2.0, 5.0, and 10.0 mg/ml (w/v) in 100% $CHCl_3$. The feeding bioassays comparing the activity of hydrolyzed versus nonhydrolyzed fractions were conducted as described for the dose–response tests with EtOAc extractables.

Only one concentration (i.e., 10 mg/ml) of aqueous extractables was bioassayed in 10 replicates as described for the dose–response test with EtOAc extractables. Forty microliters of hydrolyzed aqueous extractables redissolved

in EtO was presented as treatment, whereas, the control consisted of an equal amount of EtO.

Dose–Response Test with Fractionated EtOAc Extractables. The EtOAc extractables of green ash foliage were fractionated to determine those affecting larval feeding. Fraction A was assayed in 10 replicates of four concentrations (i.e., 0.025, 0.1, 1, and 5 mg/ml); fraction B was assayed in 10 replicates of one concentration (i.e., 3 mg/ml), due to an insufficient amount of material available; and fraction C was assayed in 15 replicates of five concentrations (i.e., 0.05, 0.1, 0.25, 1, and 3 mg/ml). The assay was the same as described for the dose–response test with EtOAc extractables. Forty microliters of either fraction was redissolved in MeOH and presented as treatment, whereas the control consisted of an equal amount of MeOH.

Quantification of Feeding Assays. The remaining portion of uneaten leaf disc was mounted between two glass microscope slides and scanned into a computer file. The computer system consisted of a Macintosh image scanner (Apple Computer, Inc., Cupertino, California), a Macintosh II computer, and a Apple Scan Ofoto image-scanning software program. Images were analyzed for remaining disc area (i.e., expressed in pixels) using the high-resolution extraction and reduction program, Collage (Image Dynamics, Madison, Wisconsin) and a Macintosh II computer (Burden and Norris, 1992).

Statistical Analyses. The remaining area of each of the two leaf discs from each feeding assay was compared using the paired t test. The remaining area of the control disc was subtracted from the remaining area of the treatment disc. A significant difference between treatment and control was determined by the $P < 0.05$ error limit. A stepwise regression was conducted with the dose–response data where the concentration, egg mass, and tree selected represented the independent variables; and the difference (the remaining area of the treatment disc − the remaining area of the control disc) represented the dependent variable. Such a method revealed concentration as the only significant ($P < 0.05$) independent variable; hence, the remaining variables were disregarded. Polynomial regression was plotted using the area-difference versus the log-transformed concentration data.

RESULTS

Chemical Analyses

Composition of Fresh Green Ash Leaves. Fresh foliage contained 2.56% EtOAc extractables, 4.93% aqueous extractables, 22.80% insoluble (80% MeOH) residue, and 69.41% water.

Acid Hydrolysis of Green Ash Extractables. TLC scanning resolved more

peaks in the acid-hydrolyzed than in the nonhydrolyzed EtOAc extractables (Figure 1). This procedure apparently yielded more entities by cleaving bonds within more complex compounds and thus releasing the increased number of simpler molecules. The breakage of glycosides into their constituent aglycones and sugars is one likely source of the increased constituents in the hydrolyzed fraction.

Fractionation of Green Ash EtOAc Extractables. Three fractions (i.e., A, B, and C) (Figure 2) jointly contained most of the peaks present in the total

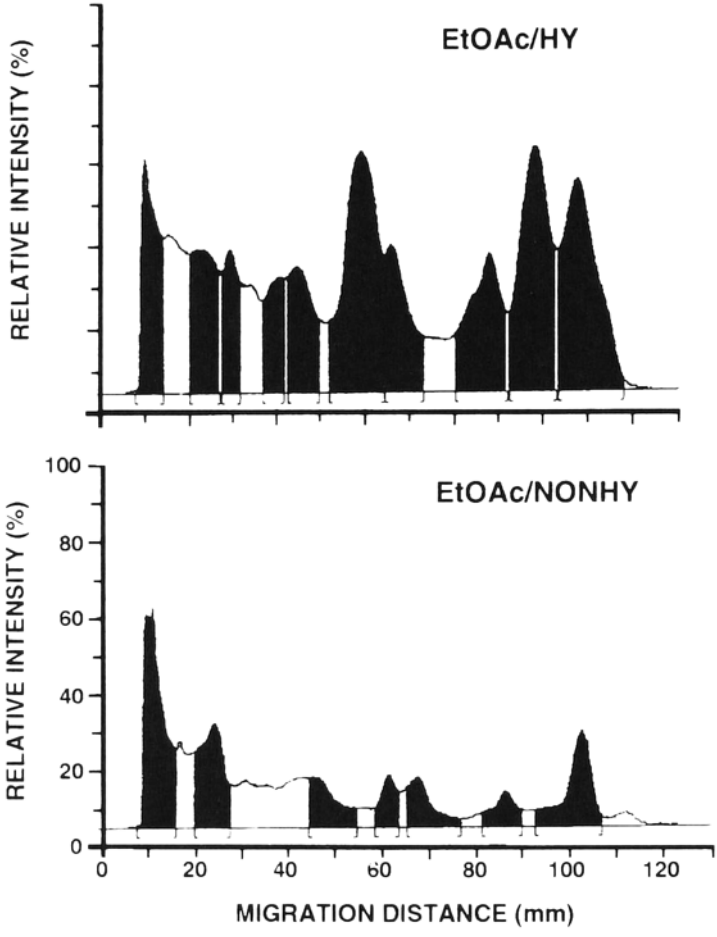

FIG. 1. HPTLC chromatograms of 10 mg/ml of hydrolyzed (EtOAc/HY) and nonhydrolyzed (EtOAc/NONHY) EtOAc extractables of green ash leaves.

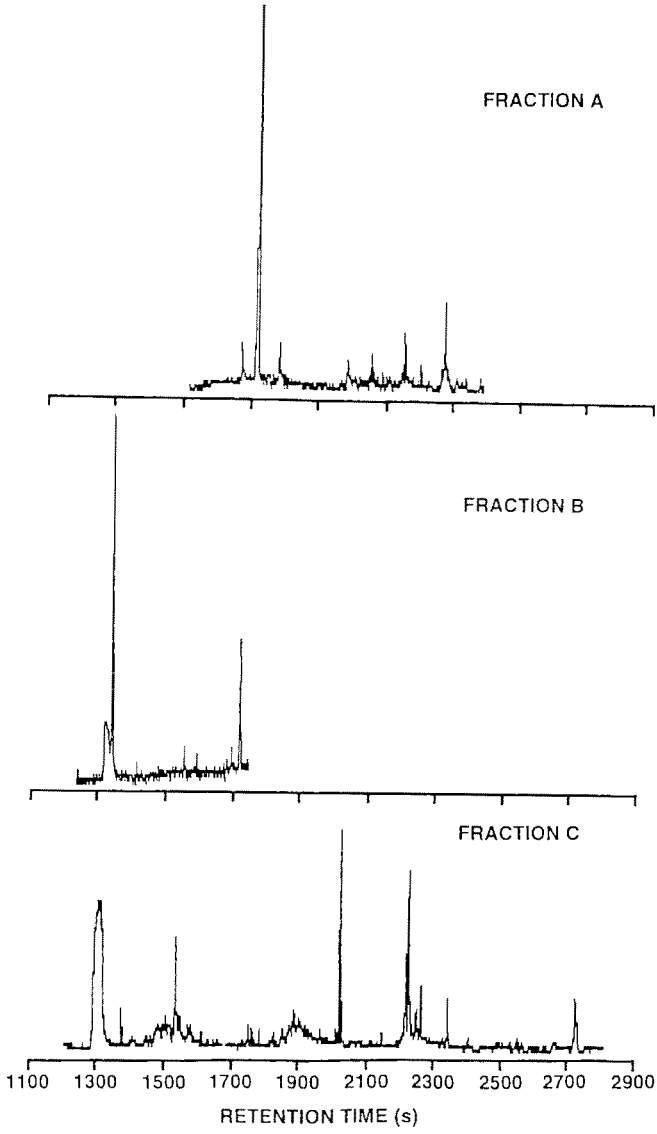

FIG. 2. GC chromatograms of three representative column-chromatographic fractions of the nonhydrolyzed EtOAc extractables. Fraction A eluted in $CHCl_3$–MeOH (9:1) on the 16 ml column; fraction B eluted in $CHCl_3$–MeOH (9:1) on the 400-ml column; and fraction C eluted in 60% aqueous MeOH on the 400-ml column.

EtOAc extractables, but each contained peaks distinct from those in the other fractions.

Feeding Bioassays

Preference Test. When both red oak and green ash leaf discs were presented to larvae in a choice assay, they showed a significant preference for red oak ($P < 0.001$). When red oak and green ash were presented in a no-choice, paired assay, larvae consumed oak significantly more ($P < 0.001$) than ash. Overall, larvae removed less than 2% of the green ash leaf disk area as compared to 52 and 53% of the red oak discs in choice and no-choice assays, respectively.

Dose-Response Test with Nonhydrolyzed EtOAc Extractables. Application of nonhydrolyzed green ash EtOAc extractables caused a significant reduction in larval feeding on the treatment (i.e., red oak leaf disc plus 40 μl of the nonhydrolyzed EtOAc extractables) as compared to the control (i.e., red oak leaf disc plus 40 μl of 100% MeOH) (Figures 3 and 4). The level of antifeedant activity depended on the dosage, e.g., dosages of 2–15 mg/ml caused a significant feeding preference for the control versus treatment at $P < 0.001$. Significantly reduced feeding was observed at 1 mg/ml ($P = 0.002$), 0.5 mg/ml ($P = 0.0043$), 0.25 mg/ml ($P = 0.011$), and 0.1 mg/ml ($P = 0.016$). Tests with

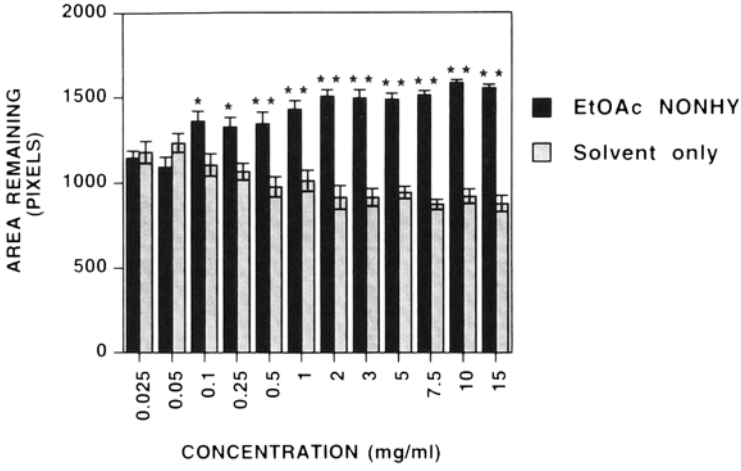

FIG. 3. Dose-response test with 12 different concentrations of nonhydrolyzed EtOAc extractables. Treatment (EtOAc NONHY) bars topped by two asterisks are significantly different from the control (solvent only) by the paired t test at $P < 0.01$; whereas those topped by one asterisk are significantly different at $P < 0.02$.

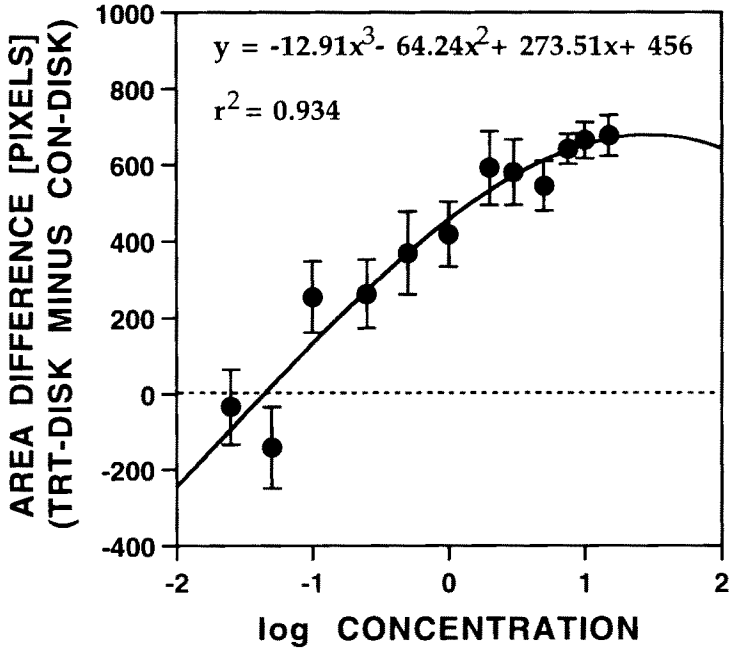

FIG. 4. Polynomial regression of the remaining disc-area difference (trt-disc minus con-disc) in pixels against the log-transformed concentrations. The higher six concentrations are not significantly different from each other (one-way ANOVA).

0.05 and 0.025 mg/ml caused insignificant effects. The six higher concentrations did not significantly differ in their antifeeding activity (one-way ANOVA).

Dose–Response Test with Nonhydrolyzed Aqueous Extractables. None of the five dosages (i.e., 0.1, 0.5, 2, 5, and 10 mg/ml) of nonhydrolyzed aqueous extractables from green ash foliage was significantly inhibitory to larval feeding (Figure 5).

Dose–Response Test with Hydrolyzed EtOAc Extractables. Acid hydrolysis of the otherwise very active EtOAc extractables destroyed antifeedant activity (Figure 6). In addition, such a procedure rendered the two highest dosages (i.e., 5 and 10 mg/ml) significantly more stimulatory to larval feeding ($P = 0.001$ and $P = 0.01$, respectively) as compared to the $CHCl_3$ control. The remaining dosages at 0.1, 0.5 and 2 mg/ml had insignificant effects.

Dose–Response Test with Hydrolyzed Aqueous Extractables. At 10 mg/ml concentration, the hydrolyzed aqueous extractables did not show significant anti-

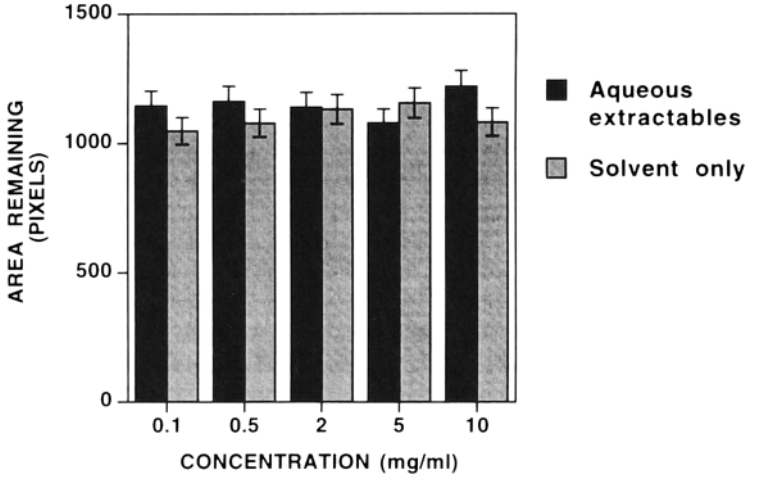

FIG. 5. Dose–response test with five different concentrations of the nonhydrolyzed aqueous extractables.

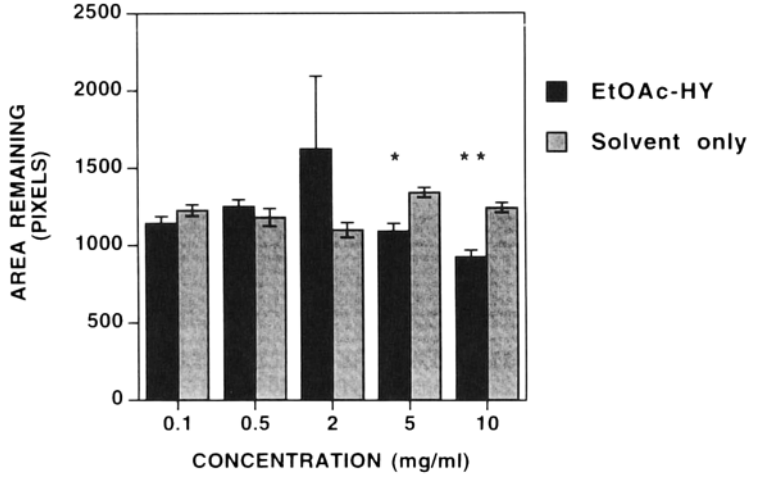

FIG. 6. Dose–response test with five different concentrations of hydrolyzed EtOAc extractables. Treatment (EtOAc-HY) bars topped by two asterisks are significantly different from the control (solvent only) by paired t test at $P < 0.001$, whereas those topped by one asterisk are significantly different at $P < 0.01$. (see text for the exact P values).

feedant activity. Doskotch et al. (1981) suggested that water-soluble compounds usually are less likely to have feeding deterrent activity.

Effects of Fractionation of Nonhydrolyzed EtOAc Extractables on Larval Feeding. Of the four assayed dosages of fraction A (Figures 2 and 7), the highest dosage at 5 mg/ml caused significant ($P = 0.0037$) feeding reduction for the treated versus untreated (control) disc. The remaining concentrations of 0.025, 0.1, and 1 mg/ml did not cause significant antifeedant activity. Fraction B caused a significant ($P = 0.001$) feeding inhibition at 3 mg/ml (Figures 2 and 7). Fraction C (Figures 2 and 7) caused an antifeedant response at 3 mg/ml ($P = 0.01$), but not at 0.05, 0.1, 0.25, and 1 mg/ml.

DISCUSSION

Insects frequently reject plants as hosts due to repellents, deterrents, and/or feeding inhibitors (Jermy, 1966; Gilbert et al., 1967; Norris, 1986, 1994). Gypsy moth larvae reject green ash as a host due to chemicals in green ash foliage. Green ash chemicals deterrent and/or inhibitory to larval feeding are contained primarily in the EtOAc extractables. These extractables caused significant reduction in larval feeding at concentrations as low as 0.1 mg/ml. Increasing the concentration above 2 mg/ml, however, did not cause significantly greater feeding reduction. The chemosensory receptors of the larvae may become saturated at relatively low levels of the EtOAc extractables.

Three representative, GS-verified, column-chromatographic fractions of EtOAc extractables contained antifeedants. Each fraction alone showed weaker antifeedant activity compared to the total EtOAc extractables. Fractionation of extractables from the tulip-tree leaves (Doskotch et al., 1981) and catalpa leaves (El-Naggar and Doskotch, 1980) similarly reduced their deterrency to gypsy moth larvae. More than one component is obviously responsible for the observed feeding-inhibitory activity of EtOAc extractables from green ash foliage. Berenbaum (1985) suggested that plant selection favors a multicomponent chemical defense, which may be directed at multiple target sites. Multicomponent activity frequently encompasses additive elements, in which the combined antifeedant activity of the components is greater than that of any individual component, and/or synergistic elements so that the combined activity is greater than the summed activity of individual components. Some synergistic chemicals (e.g., methylendioxyphenyls) may lack feeding-inhibitory activity but may suppress major detoxifying enzymes (e.g., cytochrome P-450); in this way they can significantly augment the feeding-inhibitory activity of other secondary metabolites (Berenbaum, 1985). Others (e.g., caffeine) have both feeding-inhibitory (or toxic) effects and synergize the biological activity of additional components (Nathanson, 1984). Consequently, the observed greater antifeedant activity in the EtOAc mixture, as compared to a fraction thereof, seems reasonable.

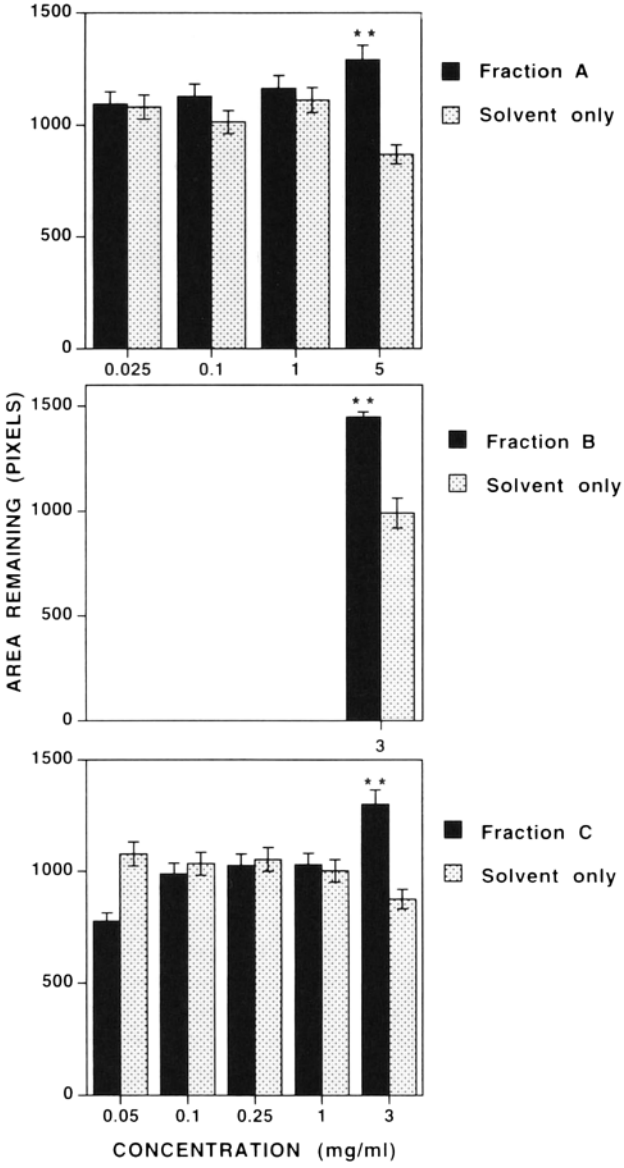

FIG. 7. Dose-response test with fractions A, B, and C of nonhydrolyzed EtOAc extractables separated by column chromatography. Treatment (fraction A, B, or C) bars topped by two asterisks are significantly differnt from the control (solvent only) by the paired t test at $P < 0.01$ (see text for the exact P values). Fraction B was assayed at only one concentration due to insufficient amount of material available.

Acid hydrolysis of EtOAc extractables destroyed antifeedant activity and yielded feeding stimulants. A reasonable explanation might be that hydrolysis broke major antifeedant components, including some glycosides and other complex molecules, into simpler chemicals. In the absence of the previously existing deterrents, existing and/or released compounds such as sugars, phenolics, and/or short-chained fatty acids may act as feeding stimulants. Structural identification of chemicals in the active EtOAc extractables from green ash foliage, their relative proportions, and the feeding-inhibitory activity of such individual components should be carried out.

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OLFACTORY RESPONSES OF *Ips plastographus maritimus* LANIER (COLEOPTERA: SCOLYTIDAE) TO INSECT AND HOST-ASSOCIATED VOLATILES IN THE LABORATORY

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Abstract—Attraction of both sexes of *Ips plastographus maritimus* Lanier to bark-phloem-xylem discs of Monterey pine, *Pinus radiata* D. Don, was demonstrated in the laboratory. Increasing concentrations of male and female volatiles trapped separately and released in a one-to-one ratio decreased attraction for both sexes combined. Attraction of both sexes to volatiles derived from males and females tunneling together in a one-to-one ratio increased with increasing concentration of extract. Attraction of males and females to male-infested discs and to trapped male volatiles increased with increasing dose of males or male extract. Attraction of males and females to female-infested discs and to trapped female volatiles was also demonstrated. The presence of females in male galleries reduced the attractiveness of infested disks to both sexes combined. Increasing numbers of females, tunneling separately from males in the same disc, reduced attraction of males, but not females. When a constant attractive dose of male volatiles was released with increasing doses of female volatiles, there was no difference in response of

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either sex when female volatiles were present compared with the response to male volatiles alone. When a constant attractive dose of male volatiles was released with increasing concentrations of volatiles derived from males and females tunneling together in a one-to-one ratio, attraction of *I. p. maritimus* decreased. Response of females was frequently higher than that of males to the same attractant source. Hence, both sexes produce an attractant, and both sexes tunneling together in the same gallery reduce attraction of males and females to an attractive dose of male attractant.

Key Words—*Ips plastographus maritimus*, Coleoptera, Scolytidae, bark beetle, *Pinus radiata*, host volatiles, aggregation pheromone, sex-specific response, attraction, interruption

INTRODUCTION

Most bark beetles (Coleoptera: Scolytidae) colonizing host trees produce pheromones that attract conspecifics of both sexes, resulting in mass attack of the host (Wood, 1982; Borden, 1985; Raffa et al., 1993). There are exceptions to this pattern of attack; for example, *Dendroctonus micans* Kug. adults are not known to produce aggregation pheromones, and do not mass attack trees (Grégoire, 1988). *Tomicus piniperda* (L.) adults also are not known to produce aggregation pheromones, but they mass attack trees (Byers et al., 1985). *Ips* spp. males initiate galleries on the host by tunneling through the bark and feeding in fresh phloem (Anderson, 1948). In all species studied, males produce a pheromone that elicits the resultant aggregation (Wood, 1982; Borden, 1985; Seybold, 1992; Raffa et al., 1993). In nature, male *Ips* spp. initiate galleries on a new host and are generally joined by one to six females (Bright and Stark, 1973). A mass attack by bark beetles concentrates the same species on host material suitable for feeding and reproduction and is dependent on pheromone production by the sex that initiates attacks on the host (Wood, 1962, 1982; Wood and Vité, 1961). For example, the aggregation pheromone released by male *Ips paraconfusus* Lanier was identified as a mixture of the three monoterpene alcohols, ipsenol, ipsdienol, and *cis*-verbenol (Wood et al., 1966; Silverstein et al., 1966), none of which elicited a response when delivered individually (Wood et al., 1967, 1968). These studies demonstrated a multicomponent attractant pheromone and synergistic effects among pheromone components (Silverstein, 1977). These pheromone components were identified using an olfactometer to assay the upwind walking behavior of *I. paraconfusus* (Wood and Bushing, 1963). More recently this upwind response was shown to be stimulated both by the presence of multiple pheromone components (chemotaxis) and by the airstream (anemotaxis) (Akers, 1989; Akers and Wood, 1989; Akers et al., 1993).

There are 27 species of *Ips* recognized in North America (S. L. Wood, 1982; Lanier et al., 1991), yet only a few species have been subjects of studies

of chemosensory behavior. *Ips plastographus* has been described based on two subspecies of geographic origin: coastal (*maritimus* Lanier) and subalpine [*plastographus* (LeConte)] (S. L. Wood, 1982; Lanier, 1970). Lanier (1970) demonstrated reduced fertility of backcross and F_2 generations between the coastal and subalpine populations, indicating a genetic difference in the populations. Both subspecies of *I. plastographus* have yielded 89–95% (+)-ipsdienol from a mixture of volatiles trapped from male-infested logs (Seybold, 1992). The attractiveness of this compound to either subspecies has not been demonstrated. However, Lanier and Wood (1975) demonstrated a strong response of female *I. p. maritimus* to male-produced frass in the laboratory. Females of three species of *Ips* from other subgeneric groups were not attracted to this frass. A pheromone response of *I. p. plastographus* males and females to male-infested logs of *Pinus ponderosa* Laws. was indicated in limited field trials (Lanier, 1970). *Ips plastographus maritimus* frequently colonizes *Pinus radiata* D. Don (Monterey pine), *P. muricata* D. Don (bishop pine), and *P. contorta* var. *contorta* Dougl. (shore pine) (Bright and Stark, 1973; S. L. Wood, 1982). *Ips plastographus plastographus* typically infests *P. contorta* var. *murrayana* Grev. and Balf. (Sierra lodgepole pine), and *P. contorta* var. *latifolia* Engelm. (Rocky Mountain lodgepole pine) (S. L. Wood, 1982).

The upwind walking response of *I. p. maritimus* to various sources of volatiles was measured in an open arena olfactometer. Volatiles originated either directly from uninfested host tissue or host tissue infested with male or female or both sexes of *I. p. maritimus* or from volatiles trapped from air passing over uninfested or infested host tissue. *I. p. maritimus* was the subject of this study because it is a potential vector of *Fusarium subglutinans* f. sp. *pini* (Storer and Dallara, 1992), the causal agent of pitch canker disease of pines. The objectives were to: (1) determine if males and females produce an attractive aggregation pheromone, and (2) determine if females produce an interruptive aggregation pheromone.

METHODS AND MATERIALS

Collection of Beetles and Host Material

Branch and stem sections of recently fallen *P. radiata* that had been colonized by *I. p. maritimus* were collected in 1994 and 1995 from Santa Cruz and San Mateo Counties in California, and placed in rearing cages (Browne, 1972). Following emergence, males and females were separated by the presence of the *pars stridens*, a stidulatory structure on the vertex of the female's head (Lanier and Cameron, 1969), and stored at 4–5°C in Petri dishes containing moist filter paper. Uninfested *P. radiata* logs for production of volatiles and for

disc assays were collected in 1994 and 1995 from Santa Cruz and Contra Costa Counties, California.

Preparation of Behavioral Stimuli

Uninfested and Infested Host Discs. Bark-phloem-xylem discs (hereafter termed discs) were prepared by cutting a fresh *P. radiata* log through the bark and phloem and into the xylem with a 5-cm cylindrical hole cutter. Discs were excised with a wood chisel and placed in clean Petri dishes. Discs were prepared either immediately before beetles were introduced or were prepared and refrigerated not more than 24 hr before beetles were introduced. Males or females were introduced into the discs in holes drilled 3 mm in diameter and 5 mm into the disk. Each drilled hole extended through the bark and phloem and onto the surface of the xylem of the disc. Beetles were confined under wire mesh that was secured on the bark surface by staples or under a gelatin capsule secured by a No. 2 insect pin. Beetles were allowed to feed in discs for approximately 24 hr before the discs were assayed.

Six disc treatments were prepared in which only one sex of *I. p. maritimus* was used. These treatments were: 0 insects; 3 or 6 males; and 3, 6, or 9 females. In addition, five disc treatments were prepared using both sexes in different sex ratios. In these cases male galleries were initiated in the discs 24 hr before introduction of females. Females were either added to male galleries or added to their own galleries in freshly drilled holes adjacent to male galleries. If females were to be added to male galleries, males were secured in their galleries by a pinned gelatin capsule, and females were added after removing the capsule. If females were introduced in different galleries from the males, fresh holes were drilled for females immediately before their introduction. In both instances the females were secured in the discs with fine wire mesh. Of the five disc treatments prepared with both sexes present, all contained six male beetles. No females were added to one disc; females were added to the male galleries in two discs in ratios of one female per male and two females per male; and females were introduced into separate galleries in two discs in ratios of one female per male and two females per male.

Chemical Extracts of Volatiles Trapped from Uninfested and Infested Host Tissue. Pinus radiata logs, approximately 30 cm in length, and with a phloem thickness of at least 2 mm, were artificially infested with male and/or female beetles. This phloem provided enough feeding material for the duration of the volatile collection period. Three logs were prepared for each of four treatments. The treatments included: 0 insects; a total of 140 males (distributed over three logs); a total of 180 females (distributed over three logs); and a total of 168 males and 168 females (distributed over three logs). Logs with either males or females tunneling alone were prepared 24 hr prior to volatile collection. Logs

containing male and female beetles tunneling together were prepared by introducing the females 24 hr after the males and commencing with volatile collection 24 hr after the introduction of the females, i.e., 48 hr after introduction of the males.

To trap host- and insect-produced volatiles, beetle-colonized logs were placed into 19-liter glass carboys for approximately 168 hr. Air was drawn through activated charcoal into these chambers and passed through Porapak Q (50–80 mesh, Supelco Inc., Bellefonte, Pennsylvania) to trap compounds volatilizing from the insect-colonized and uncolonized host tissue (Seybold, 1992). The Porapak was extracted with 350 ml of pentane, and the extract was concentrated by rotary evaporation to produce a 10-ml stock solution of crude extract. The stock solution was stored at −35°C.

Behavioral Assay

An open arena olfactometer (Figure 1) was used to assay the attraction of walking *I. p. maritimus* to various olfactory stimuli. The experimental method

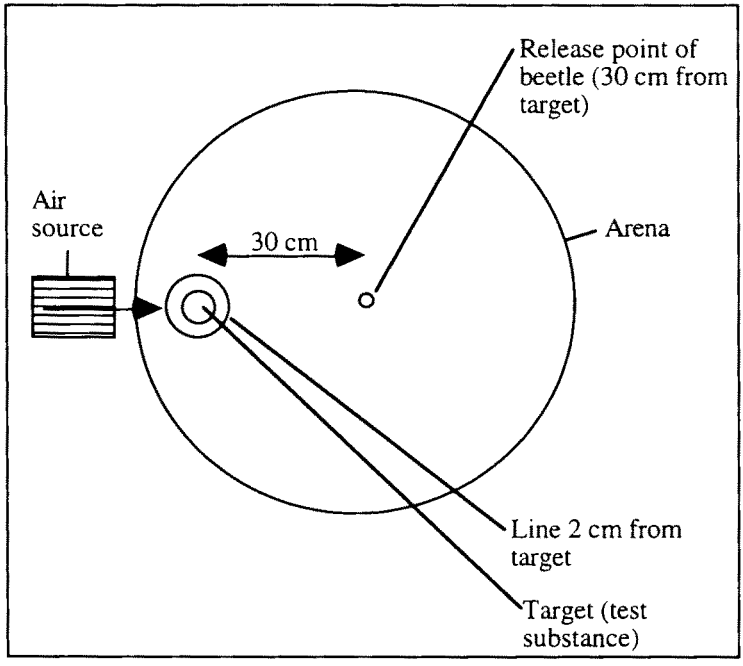

FIG. 1. Olfactometer with arena, air source and direction, target, and release point indicated.

used was adapted from Wood and Bushing (1963) as modified by Seybold (1992). Airflow into the arena at 0.76 ± 0.2 m/sec from a fan was maintained with a rheostat. A receptacle just downwind in the airstream was designated as the target and contained an infested or uninfested disk of *P. radiata* or a chemical extract. The olfactometer chamber was prepared before each assay by cleaning the arena with 70% ethanol and covering the arena with disposable newsprint sheets. The ethanol was evaporated from the arena under maximum air speed for a minimum of 20 min before the arena was covered with newsprint. The disposable sheets allowed the test beetles to maintain a secure footing and reduced the possibility of cross-contamination between treatments. A circular pencil mark was drawn on the paper 2 cm immediately preceding the target. Individual beetles were placed facing the target, 30 cm downwind of the target. A beetle passing the 2-cm mark within a 2-min time limit was considered to have responded to the test substance in the target and was scored as a positive response. Whenever possible, 30 individual males and females were exposed to a treatment, except where the supply of beetles was limited. All beetles were examined prior to the assay to ensure that they were alive, with intact legs and antennae.

Stock solutions of extracts were designated as having a concentration of 10^0, and dilutions were prepared from the extracts to produce concentrations of 10^{-1} to 10^{-6}. The diluted extracts were stored long-term at 4°C. During the assays the diluted extracts were placed on Dry Ice to reduce evaporation. To assay each beetle against a dilution of trapped volatiles, extracts were loaded into 5-μl micropipets (Drummond Scientific Co., Broomall, Pennsylvania). The micropipet was mounted on a foil-covered holder and placed under the airstream in the olfactometer. Newly loaded micropipets were used for each beetle.

Increasing concentrations of male, female, and male and female extracts were tested to determine their attractiveness to individual male and female *I. p. maritimus*. To simulate males and females tunneling separately in a ratio of one to one, extracts from each sex were released at simultaneously increasing concentrations. To investigate possible interruptive effects, a known attractive dose of male extract (10^{-2}) was released with either increasing doses of female extract or increasing doses of extracts from males and females tunneling together.

Statistical Analyses

Hierarchical log-linear analyses (Zar, 1984) were conducted to determine the relationship among three variables: response, dose, and sex. Response was determined from the number of beetles reaching the target within the 2-min time limit. Dose was the number of beetles in the host disc or the concentration of the extract of volatiles. Sex was the gender of the responding beetle. Analyses were carried out using SPSS Release 6.0 (SPSS Inc., Chicago, Illinois, 1993), which produces a best-fit final model for each data set.

Where the two-way interaction between dose and response was significant, sexes were grouped for orthogonal pairwise comparisons between doses by calculating the G statistic for each comparison (Sokal and Rohlf, 1981). If the two-way interaction between sex and response was significant, doses were grouped for the purpose of reporting the response by each sex. If the final model included a three-way interaction, pairwise comparisons were computed by sex for response versus dose. Comparisons were made between the highest and lowest doses. In each case, the comparisons were designed to contrast response to higher doses with response to lower doses.

For the data from the volatiles derived from beetle-infested logs, Spearman rank correlations were calculated to compare the ranking of the dose and the ranking of the response. This was done to determine whether the rank of the dose correlated significantly with the rank of the response. The significance of the R_s values calculated were assessed using tables of R_s with a low N (Siegel, 1956). If the correlation coefficients were significant, pairwise comparisons were not calculated. When the factor of dose was a component of the best-fit model, orthogonal pairwise comparisons were carried out as described previously.

RESULTS

Uninfested and Infested Host Discs

Experiment 1: The attraction of male and female *I. p. maritimus* was significantly greater when a host disc was present, than when it was not (dose * response, $P < 0.0001$) (Table 1, A). Of those beetles exposed to uninfested host discs, 40.8% approached the target, while 25.0% of the beetles approached the target when the host was not present. Overall, females were more likely to approach the target than males (38.0% vs. 28.2%) (sex * response, $P = 0.024$).

Experiment 2: The attraction of male and female *I. p. maritimus* to host discs infested with males was dependent on the number of males tunneling in the host (dose * response, $P < 0.001$) and increased with increasing numbers of males in the host (Table 1, B). Response by both sexes combined was greater when the host disc contained three or six males than when the host disc contained no beetles (66.7% vs. 30.0%, $G = 21.83$, 1 df, $P < 0.01$). More beetles were attracted to host discs containing six males than host discs containing three males (78.3% vs. 55.0%, $G = 7.36$, 1 df, $P < 0.05$). Response did not depend on the sex of the responding beetle (Table 1, B).

Experiment 3: Male and female *I. p. maritimus* were attracted to host discs containing females (dose * response, $P = 0.003$) (Table 1, B). Attraction of both sexes combined was greater when the host disc contained three, six, or nine female beetles than when the host disc contained no beetles (55.0% vs.

TABLE 1. PROPORTIONS OF MALE AND FEMALE *Ips plastographus maritimus* ATTRACTED TO *Pinus radiata* DISCS CONTAINING MALE AND/OR FEMALE BEETLES[a]

Exp.		Interactions from log linear model ($P < 0.05$)	Sex of responding beetle	Host disc			
				Absent	Present		
A. Uninfested disks							
1		Dose * response	Male	0.20b	0.35c		
		Sex * response	Female	0.29c	0.46d		
B. Single sex disks							
	Sex of beetles in disc			Number of beetles per disc			
				0	3	6	9
2	Male	Dose * response	Male	0.30	0.70	0.77	—
			Female	0.30	0.40	0.80	—
3	Female	Dose * response	Male	0.23	0.47	0.63	0.57
			Female	0.37	0.47	0.57	0.60
C. Multiple sex disks							
	Male and female gallery			Number of females/male			
				0	1	2	
4	Same	Dose * response	Male	0.57	0.53	0.30	
		Sex * response	Female	0.83	0.57	0.50	
5	Different	Dose * sex * response	Male	0.58	0.63	0.13	
			Female	0.83	0.60	0.80	

[a] Paired comparisons based on the outcome of the log-linear analyses are reported in the text. N = 30 except; b, N = 100; c, N = 120; d, N = 130.

30.0%, $G = 11.42$, 1 *df*, $P < 0.01$). There was no significant difference in attraction between host discs containing three, six, or nine females. Response did not depend on the sex of the responding beetle (Table 1, B).

Experiment 4: When exposed to host discs that contained one or two females tunneling in male galleries, the combined response of males and females decreased compared with the response to discs that contained just males (47.5% vs. 70.0%, $G = 8.29$, 1 *df*, $P < 0.01$) (dose * response, $P < 0.004$). There was no significant difference in attraction when beetles were exposed to either one or two females in a male gallery. Overall response of females was higher than males (63.3% vs. 46.7%) (sex * response, $P = 0.024$) (Table 1, C).

Experiment 5: When the number of females tunneling separately from males increased from one to two, male response decreased significantly while female

response did not (Table 1, C). Response was dependent on both dose and sex (dose * sex * response, $P < 0.001$). Male response was lower when two females were tunneling side by side with one male than when one female was tunneling side by side with a male (13.3% vs. 63.3%, $G = 16.45$, 1 *df*, $P < 0.01$); female response remained unaffected.

Chemical Extracts of Volatiles Trapped from Uninfested and Infested Host Tissue

Experiment 6: The response of male and female beetles to the stock solution and to the 100× dilution of the crude extract of volatiles from uninfested logs of *P. radiata* did not significantly differ from the response to pentane (43.3%, 48.3%, and 41.7% response, respectively; Table 2). None of the interactions involving these host volatiles were significant.

Experiment 7: The response of male and female *I. p. maritimus* to extracts from *P. radiata* logs infested with males increased as the dose of volatiles increased (Table 2). Response was dependent on dose (dose * response, $P < 0.001$), and the ranking of response was positively correlated with the ranking of the dose ($R_S = 1.00$, $N = 6$, $P < 0.01$). Overall, female attraction was greater than male attraction (48.9% vs. 37.2%; sex * response, $P = 0.025$).

Experiment 8: The response of male and female *I. p. maritimus* to extracts from *P. radiata* logs infested with females was dependent on the dose of the volatiles (Table 2) (dose * response, $P < 0.001$). Although the ranks of dose and response did not correlate, orthogonal comparisons indicated that the greatest attraction occurred before the highest dose. Overall response of both sexes combined was higher to extracts from female beetles tunneling in the host than to pentane alone (47.1% vs. 25.0%, $G = 9.69$, 1 *df*, $P < 0.05$). Attraction to the two highest doses of female extract tested (10^{-2}, 10^{0}) was lower than to the two lowest doses (10^{-4}, 10^{-3}) (35.8% vs. 62.5%, $G = 14.52$, 1 *df*, $P < 0.01$). There was no significant difference in attraction between the two lowest doses of extract. The highest dose (10^{0}) was less attractive than the next highest dose (10^{-2}) (15.0% vs. 56.7%, $G = 24.43$, 1 *df*, $P < 0.01$). Overall, females were more likely than males to respond to any dose of female extract (48.1% vs. 36.1%; sex * response, $P = 0.045$).

Experiment 9: Male and female *I. p. maritimus* were attracted to extracts from logs in which males and females tunneled in a one-to-one ratio in the same gallery, and attraction increased with increasing dose (Table 2). Response was dependent on dose (dose * response, $P < 0.001$), and there was a positive correlation between the ranks of dose and response ($R_S = 1.00$, $N = 5$, $P < 0.05$). Response did not depend on the sex of the responding beetle.

Experiment 10: Response of males and females to male-derived extracts and female-derived extracts released simultaneously in a one-to-one ratio gen-

TABLE 2. PROPORTIONS OF MALE AND FEMALE *Ips plastographus maritimus* ATTRACTED TO INDIVIDUAL EXTRACTS OF VOLATILES FROM *Pinus radiata* LOGS CONTAINING MALE OR FEMALE BEETLES OR FROM LOGS CONTAINING BOTH MALE AND FEMALE BEETLES IN THE SAME GALLERY[a]

Exp.	Sex of beetles in log from which extract was prepared	Interactions from log-linear model ($P < 0.05$)	Sex of responding beetle	Concentration of extract						
				0 (pentane)	10^{-6}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{0}
6	No beetles	None	Male	0.40				0.53		0.40
			Female	0.43				0.43		0.47
7	Male	Dose * response	Male	0.27		0.23	0.23	0.53	0.43	0.53
		Sex * response	Female	0.23		0.33	0.50	0.43	0.73	0.70
8	Female	Dose * response	Male	0.27		0.46b	0.60c	0.43		0.17
		Sex * response	Female	0.23		0.70	0.80c	0.70		0.13
9	Male & Female	Dose * response	Male	0.17	0.23	0.47		0.53		0.80
			Female	0.20	0.20	0.20		0.57		0.57

[a]Paired comparisons based on the outcome of the log-linear analyses are reported in the text. $N = 30$ except: b, $N = 28$; c, $N = 15$.

erally decreased with increasing concentration of the extracts (Table 3). Response was dependent on dose (dose * response, $P < 0.001$). There was no significant correlation between the ranks of dose and response. Attraction was higher to all combinations of beetle-derived extracts than to pentane alone (60.1% vs. 33.3%, $G = 14.37$, 1 *df*, $P < 0.01$). The three lowest doses were more attractive than the two highest doses of volatiles (65.7% vs. 52.1%, $G = 5.37$, 1 *df*, $P < 0.05$), and the highest of the three lower concentrations (10^{-2}) was less attractive than the other two lowest concentrations (10^{-4}, 10^{-3}) (53.3% vs. 72.3%, $G = 6.05$, 1 *df*, $P < 0.05$). There were no differences in response between the two highest or the two lowest concentrations. Response did not depend on the sex of the responding beetle (Table 3).

Experiment 11: When a fixed attractive dose of male volatiles was released with increasing doses of female volatiles, response was dependent on the dose of female volatiles (dose * response, $P = 0.025$) (Table 3). There was no significant correlation between the ranks of dose and response. There was no difference in the combined response to treatments in which female volatiles were present, compared to the male volatiles alone, but attraction was lower at the lowest dose of female volatiles (10^{-4}) than at the next highest dose (10^{-3}) (55.0% vs. 78.3%, $G = 7.36$, 1 *df*, $P < 0.05$). No other doses were significantly different. Regardless of dose, females were more likely to reach the target than males (75.0% vs. 59.4%; sex * response, $P = 0.002$).

Experiment 12: When a fixed attractive dose of male volatiles was released with increasing concentrations of volatiles derived from males and females tunneling together in a one-to-one ratio, attraction of *I. p. maritimus* decreased (Table 3). Response was dependent on dose (dose * response, $P = 0.037$), and the ranks of response and dose were negatively correlated ($R_s = -0.90$, $N = 5$, $P < 0.05$). The overall level of attraction of females was higher than for males (64.7% vs. 50.0%; sex * response, $P = 0.012$).

DISCUSSION

Tunneling male *I. p. maritimus* elicited attraction in the laboratory (experiment 2). This result supports an earlier study where *I. p. maritimus* females were strongly attracted to male frass or ethanol extracts of frass (Lanier and Wood, 1975). The presence of a male aggregation pheromone in *I. p. maritimus* is supported by earlier investigations of several *Ips* spp. (Wood et al., 1966; Lanier, 1970; Lanier and Wood, 1975; Birch et al., 1980; Wood, 1982; Borden, 1985; Raffa et al., 1993). In addition, a dose–response relationship for extracts from male-infested logs was demonstrated for *I. p. maritimus* (experiment 7).

This study suggests that female *I. p. maritimus* also produce an attractant pheromone (experiments 3 and 5), but at higher concentrations female volatiles appear to decrease in attractiveness (experiment 8). This decrease in attraction

TABLE 3. PROPORTIONS OF MALE AND FEMALE *Ips plastographus maritimus* ATTRACTED TO COMBINATIONS OF EXTRACTS OF VOLATILES FROM *Pinus radiata* LOGS CONTAINING MALE OR FEMALE BEETLES[a]

Exp.	Treatment	Interactions from log-linear model ($P < 0.05$)	Sex of responding beetle	Concentration of female extract[b]						
				0	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{0}
10	Male extract + female extract (1 : 1)	Dose * response	Male	0.33		0.60	0.73	0.60	0.40	0.43
			Female	0.33		0.76b	0.82c	0.47	0.52d	0.73
11	Fixed attractive dose of male extract (10^{-2}), with varing doses of female extract	Dose * response	Male	0.53		0.43	0.73	0.73	0.57	0.57
		Sex * response	Female	0.73		0.67	0.83	0.83	0.77	0.64e
12	Fixed attractive dose of male extract (10^{-2}), with varying doses of extract from males and females tunneling together	Dose * response	Male	0.63	0.53		0.63	0.37		0.33
		Sex * response	Female	0.73	0.57		0.67	0.67		0.56f

[a] Paired comparisons based on the outcome of the log-linear analyses are reported in the text. $N = 30$ except: b, $N = 25$; c, $N = 27$; d, $N = 23$; e, $N = 22$; f, $N = 16$.

[b] In experiments 11 and 12, a fixed attractive dose of male extract (10^{-2}) is always present.

suggests that at high levels, pheromone components produced by females may prevent overexploitation of the host. *Ips latidens* (LeConte) females also produce a pheromone (Seybold, 1992), but laboratory studies indicate that it is not an attractant at any dose. Female *I. latidens* pheromone reduces the response to the male-produced attractant (Seybold, 1992). This does not appear to be the case with female *I. p. maritimus* (experiment 11). In nature, *Ips* spp. females generally tunnel only when the male is present inside the common gallery system. This joining of males and females may influence the production of pheromone compounds in both sexes. An attractant pheromone produced by an *Ips* spp. female has not been previously reported, but attractant pheromone components are produced by both sexes in several species of *Dendroctonus* (Wood, 1982).

When male and female *I. p. maritimus* were introduced into separate galleries, only male response was interrupted (experiment 5). In nature, these volatiles would be produced only after joining has occurred. An exception to this behavior occurs in *I. p. plastographus*, in which males and females have been observed in separate galleries in Sierra lodgepole pine. This rare observation has been attributed to the overwintering behavior of this species (Johnson, 1954; Lanier, 1967). *Ips plastographus maritimus* male galleries containing one or two females were less attractive to both males and females than discs containing only tunneling males (experiment 4). Attraction was also reduced when increasing doses of extracts from males and females tunneling together were released with an attractive doses of male extract (experiment 12). The release of simultaneously increasing doses of male extract and female extract caused a reduced response at the highest concentration (experiment 10). However, in the presence of an attractive concentration of male extract, increasing concentrations of female-produced extract did not cause a decreased response for males or females (experiment 11) as was observed for males when males and females were tunneling side by side in host discs (experiment 5). Further work is required to determine why the female extract did not reduce the male response. Varying the dose of both male and female attractant to produce an entire response surface (e.g., Akers and Wood, 1989; Akers et al., 1993) may reveal interruptive activity as observed with males and females tunneling side by side (experiment 5). Chemical identification studies are needed to determine whether or not the male interruptant(s) produced by males and females tunneling side by side or together are the same. Clearly, joining is required for female interruption (c.f. experiments 4 and 5).

Responses of males and females to extracts of volatiles produced by males and females tunneling together (in a one-to-one ratio) increased with increasing concentration of extracts (experiment 9). These attractants also function as interruptants when released with an attractive dose of male extract (experiment 12).

Further studies are needed to determine attractant/interruptant activity of volatiles produced by one-to-two and one-to-three (male to female) sex ratios.

In *I. p. maritimus*, mating is not required to produce interruption (experiments 5 and 10). Borden (1967) showed that attraction of *I. paraconfusus* females to excised guts and frass in the laboratory decreased as the number of females joining males increased. However, his experiments did not establish whether mating was necessary to produce this effect after joining occurred. Byers (1981) showed that logs infested with male and female *I. paraconfusus* in a ratio of 1:3 reduced attraction of males, but not females, to male-infested logs in the field. These results support, in part, our experiment 12, in which an extract of volatiles produced by males and females tunneling together elicited a decreased response by males to an attractive dose of male-produced volatiles.

Stridulation by females (Barr, 1969) may stimulate male-produced, interruptive volatiles, as has been shown for *Dendroctonus pseudotsugae* Hopkins (Rudinsky, 1969). Males of *D. brevicomis* LeConte also produce attractive (frontalin) and interruptive (verbenone) compounds that regulate the response of the attacking population to a female-produced attractant (*exo*-brevicomin) (Byers et al., 1984). In *Dendroctonus* spp., females initiate galleries on the host, and only one male joins the female gallery. Reduced attraction after the males join the female, as shown for *D. ponderosae* Hopkins (Edson, 1978), may prevent overexploitation of the host, therefore increasing the probability of brood survival.

Female *I. p. maritimus* response to all pheromone sources was greater than male response. *Tomicus piniperda* (Byers, 1989), *I. pini* (Birch et al., 1980), *I. paraconfusus* (Wood, 1962; Borden, 1967; Seybold, 1992), *I. confusus* LeConte (Birch et al., 1977), and *I. latidens* (Wood et al., 1967, Seybold, 1992), have also shown this sex-specific response in the laboratory. Polygamous joining increases the chances for male reproductive success. The lowered attraction of males to male galleries and to male galleries joined by females increases the chances for colonization to occur on nearby uninfested areas on the same tree and on nearby trees, and thus prevents overutilization of host material.

This study demonstrates that *I. p. maritimus* produces male and female aggregation pheromone components and that the presence of one or two females in the male gallery decreases attraction of both sexes combined. Identification of the male and female attractant pheromone components would provide a useful technique for trapping *I. p. maritimus* in order to determine the phoresy (capacity of insects to carry fungus) of *Fusarium subglutinans* f. sp. *pini* (causal agent of pitch canker disease), a pathogen first discovered in California in 1986 (McCain et al., 1987). This bark beetle is known to carry this pathogen (Storer and Dallara, 1992), but, unlike *I. paraconfusus* and *I. mexicanus* (Hopkins) (Fox et al., 1991), its importance as a vector of the fungus is unknown. Identification of the female- and male-produced interruptants of male and female attraction to

male-produced pheromones may also provide a useful technique for preventing attacks by *I. p. maritimus* and perhaps other *Ips* spp.

Although *I. p. maritimus* were attracted to cut discs of *P. radiata* (experiment 1), they were not attracted to either of two doses of volatiles trapped from uninfested logs of *P. radiata* (experiment 6). The lack of attraction of the extract may have occurred because the doses of host volatiles used for assay were too concentrated or the compounds in the discs that elicited attraction may not have been trapped from the logs by Porapak Q. In some scolytids attraction to the host is a subtle effect, requiring extensive sampling (Moeck et al., 1981). *Ips latidens* and *Hylastes gracilis* LeConte have been shown to respond in the field to host volatiles from lodgepole pine in British Columbia (Miller et al., 1986; Miller and Borden, 1990). *Ips grandicollis* (Eichh.) responds to physiologically weakened *Pinus taeda* L. (Werner, 1971, 1972). Laboratory studies by Gast et al. (1993) suggested a host response by *I. pini*, although a response to air alone was not presented to control for the low response (9.8–13.9%) to host odors. The attraction of *I. p. maritimus* to *P. radiata* increases the likelihood that beetles will find a suitable host soon after dispersal. Less exposure time to environmental hazards will increase the likelihood of survival and reproduction (Wood, 1982).

Further work is needed to verify host response by *I. p. maritimus*. Although there was a significant attraction to host discs, the response to trapped volatiles was not demonstrated. Additional work is needed to determine if the response of *I. p. maritimus* to host volatiles can be interrupted. Such a tactic would prevent production of aggregation pheromones and thereby may be more effective in preventing host colonization than interrupting pheromone production during host colonization.

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COMPARATIVE STUDY OF HERMIT CRAB RESPONSES TO SHELL-RELATED CHEMICAL CUES

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Abstract—The responses of Australian hermit crabs to two types of shell-related odor cues were compared. Introduction of gastropod snail odor elicited an increase in grasping of shells in all four species tested (*Clibanarius infraspinatus*, *C. taeniatus*, *C. virescens*, and *Diogenes avarus*). However, the magnitude of response in the case of *C. virescens* was significantly lower than the responses of individuals of the other species. Individuals of *C. infraspinatus* responded to introduction of conspecific hemolymph with an increase in both locomotion and the frequency of grasping of shells. *C. infraspinatus* also responded to the hemolymph of the other three species, and there were no differences in the magnitude of the responses depending upon the source of the hemolymph. When individuals of *C. infraspinatus* were exposed to snail odor and conspecific hemolymph at the same time, the responses were indistinguishable from those shown to hemolymph alone.

Key Words—Hermit crabs, odors, snail odor, hemolymph, *Clibanarius* spp., *Diogenes avarus*, gastropods, crustaceans.

INTRODUCTION

The variety of ways that hermit crabs find and obtain appropriate gastropod shells for occupation is impressive. The use of chemical cues to locate potential shells for occupation has been particularly well studied in recent years (Hazlett and Herrnkind, 1980; Kratt and Rittschof, 1991; Rittschof, 1980a,b; Rittschof et al., 1990). Two main categories of shell-related cues have been investigated:

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cues emanating from the degradation of snail flesh and cues associated with conspecific hemolymph. The latter would be released in the environment in the event of the death or injury of another hermit crab, which may indicate to hermit crabs that a shell is available for occupation. Injury-related cues work both intraspecifically to stimulate the investigation of the shells of other hermit crabs and to a lesser extent across species (Rittschof et al., 1992). Odor cues from damaged conspecifics are effective for both aquatic and terrestrial species (Small and Thacker, 1994; Thacker, 1994).

Cues from the degradation of snail flesh stimulate both an increase in locomotory behavior and an increase in the frequency of grasping of other shells by hermit crabs (Hazlett, 1979; McLean, 1974; Rittschof et al., 1992). At least in *Clibanarius vittatus*, the grasps are very brief unless the shell grasped is the same species as that indicated by the odor and is unoccupied (Hazlett and Rittschof, 1996). The response to conspecific hemolymph is also an increase in locomotion and frequency of grasping behavior, but with this cue the grasps tend to be longer in duration and more readily involve full investigation of a shell even if occupied (Hazlett, 1996a). Responses to both cues tend to be stronger when crabs occupy shells that are either too small or too large (Katz and Rittschof, 1993; Rittschof et al., 1992).

The vast majority of the reports on responses to these types of chemical cues have been on species common in the intertidal areas along the east coast of the United States, especially *Clibanarius vittatus*, as well as *Pagurus pollicaris* and *Pagurus longicarpus* (McLean, 1974; Rittschof, 1980a,b). While the responsiveness of these species to cues is well established, it is of interest to compare the types of responses shown by those species to that of other species of hermit crabs to examine the generality of this category of shell-acquiring behavioral responses and to look for differences in responses among species and to relate them to either phylogeny or ecological differences. In addition, the responses of individuals of one species of hermit crab to the hemolymph from a series of sympatric Australian species were studied to look at the question of what factors limit the degree of cross-species reactivity. Finally, the responses of individuals of *Clibanarius infraspinatus* were recorded when two inputs were presented simultaneously and compared to those recorded when single inputs were presented to examine the nature of stimulus integration.

METHODS AND MATERIALS

Observations were made in the laboratory at James Cook University, Townsville, Queensland, Australia, during February and March 1995. The behavior of crabs was recorded before and after exposure to potential chemical cues from either snails, hermit crabs, or both. The crabs used were all intertidal

species in the Family Diogenidae, and all were collected at Rowes Bay, Townsville, Queensland, and transported to the laboratory. Crabs of a particular species were held in aquaria prior to being placed in round plastic dishes for observations of behavior patterns.

The species tested with snail odor (see below) were *Clibanarius infraspinatus*, *Clibanarius taeniatus*, *Clibanarius virescens*, and *Diogenes avarus*. *C. virescens* is found in rocky intertidal areas, while the other three species are from sandy, mudflat areas that are often divided into narrow pools at low tide (Haig and Ball, 1988). In addition to tests with snail odors, responses of individuals of *Clibanarius infraspinatus* were recorded following addition of conspecific hemolymph, hemolymph from other species of hermit crabs, and a combination of conspecific hemolymph and snail odor.

In all tests, sets of four crabs at a time were placed in an observation dish (21 cm in diameter) filled to a depth of 2.5 cm with seawater and the bottom covered with about 1 cm of clean sand. These opaque dishes were rinsed in running seawater between each trial. Imaginary lines across the dish connecting marks on the sides of the dishes divided the bottom surface into four quarters. After a 1-min acclimation period, I recorded the number of lines crossed by the four crabs during a 3-min observation period and the number of times any crab grasped the shell of another crab with its walking legs. The grasps were categorized as long (lasting more than 1 sec) or brief (lasting about 1 sec) because work with another species of hermit crab indicated that different length grasps were executed depending upon the type of stimulus detected.

Each set of four crabs was tested first with a control solution of seawater added to the observation dish, and then test solution(s) were added and records of locomotion and grasping were made for 3 min. For each test and control solution, between 16 and 20 replicate sets of four crabs were utilized for each species.

Snail odor was prepared by the freeze–thaw technique of Rittschof (1980b), utilizing 10 g of snails in 200 ml of seawater. The snail flesh in seawater was frozen overnight and allowed to thaw to room temperature for use on a day of testing. The supernatent, which will be referred to as snail odor, was drawn off as needed for tests. While this solution contained a variety of gastropod products, Kratt and Rittschof (1991) have demonstrated that the shell-related responses to such a solution are the same as the responses to solutions generated by the action of digestive enzymes from snail predators acting on snail muscle. The snails used were a mixture of *Thais leuteostoma* and *Morula marginalba*. These are common intertidal gastropods in Rowes Bay, and the shells are occupied by some individuals of all the species tested. In all snail odor tests, 1 ml of the test solution was introduced into the observation dish.

Hermit crab odor solutions were prepared by crushing 3.2 g of the appropriate species of hermit crab and mixing the flesh with 50 ml of seawater.

Rittschof et al. (1992) demonstrated that the shell-related behavior patterns shown by hermit crabs exposed to damaged conspecific individuals were in response to peptides in conspecific hemolymph. Therefore, although the preparations used in this study contained a variety of hermit crab chemicals, the preparations will be referred to as hemolymph. Because the hermit crab species differ in size, the number of individuals used to make up the solutions varied: one individual of *C. infraspinatus*, two of *C. taeniatus*, six of *C. virescens*, and 18 of *D. avarus*. One milliliter of the test solution was introduced into the observation dish for each test.

The responses of individuals of *C. infraspinatus* to the introduction of the conspecific hemolymph solution in their field-collected shells was very weak compared to the responses shown by individuals of other species tested (*C. vittatus*) (Hazlett, 1996a; Rittschof et al., 1992). Therefore, to increase the shell-related motivation of crabs, the shell apertures of 30 individuals were clipped back with a pair of pliers to make the shells smaller than desired by those size crabs (Katz and Rittschof, 1993). These crabs with clipped shells were tested with conspecific hemolymph, and these same individuals were utilized in tests with all remaining hermit crab preparations. In addition, these crabs were tested with a combination of snail odor and conspecific hemolymph introduced simultaneously.

Initially to look for an effect of the introduction of potential chemical cues, the behavioral scores during the control period (seawater introduction) and test period were compared by paired t tests. In order to compare the magnitude of the response of the various species, the difference between scores on locomotion and grasping during the control and test period was calculated for each replicate, and these scores were compared across species by ANOVA. In some cases, individual contrasts (Wilkinson, 1988) were used to determine which treatments or species were different from one another.

RESULTS

Individuals of all four of the species tested with snail odors responded to introduction of the snail solution, but there were differences among the species. *Diogenes avarus* showed an increase in locomotion ($t = 3.2$, $df = 19$; $P = 0.005$), but none of the other species showed a change in the rate of locomotion, although there was a trend toward increased locomotion for *C. virescens* ($t = 2.06$, $df = 15$; $P = 0.057$) (Figure 1). All four species showed a significant increase in the number of brief grasps and in the number of long grasps executed following the introduction of the snail odor (Figure 1). However, there were differences between the species in the frequency of this grasping response to snail odor introduction (Figure 2). When the differences between test and control

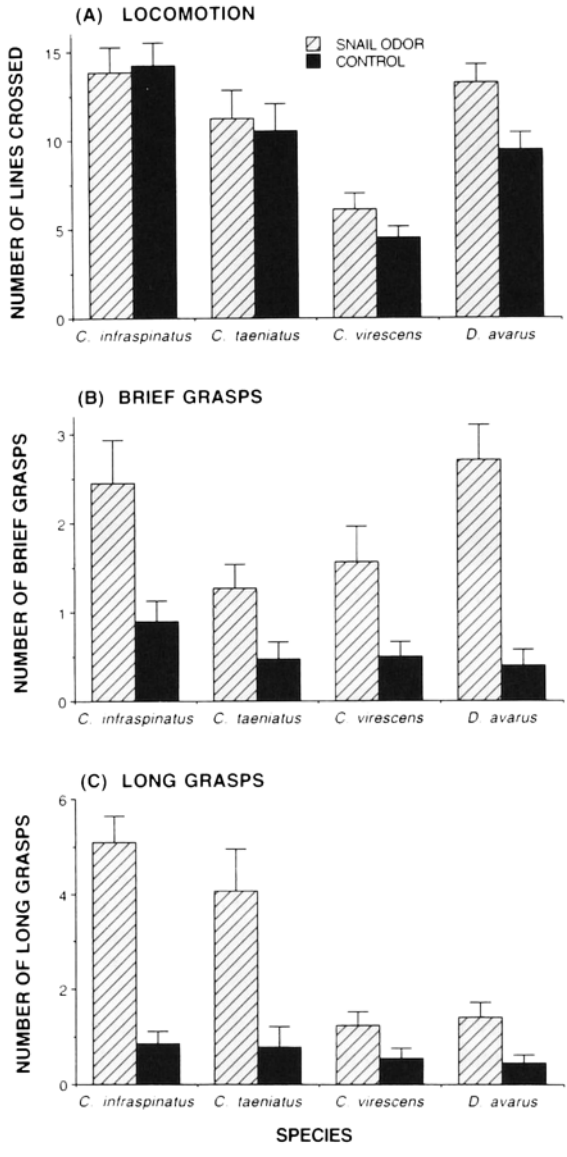

FIG. 1. Scores for behavioral parameters (means + standard errors) during introduction of seawater control (black bars) and snail odor (crosshatched bars) for four species of Australian hermit crabs.

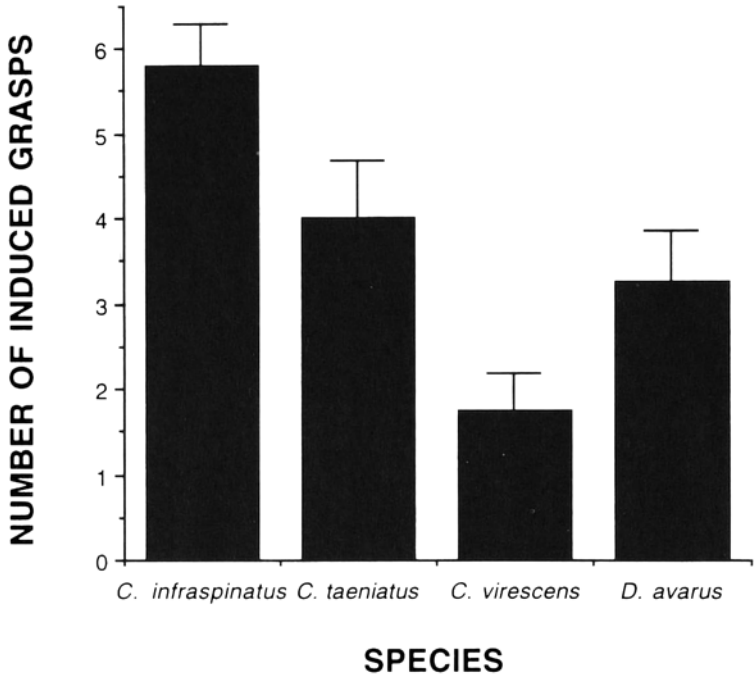

FIG. 2. Difference (mean + standard error) between number of grasps by individuals of four species of hermit crabs during control water and snail odor introductions.

periods for brief grasps and long grasps were summed for each replicate and the species compared, there were significant differences (ANOVA $F = 20.3$, $dp = 4, 78$; $P < 0.001$). This difference apparently is a result of the weaker response (although statistically significant) of individuals of *C. virescens* to snail odor (individual comparisons of *C. taeniatus* vs. *C. virescens* yielded $F = 29.6$, $dp = 1, 78$; $P < 0.001$; *C. virescens* vs. *D. avarus*, $F = 5.23$, $dp = 1, 78$; $P = 0.023$).

There were very weak responses of the individuals of *C. infraspinatus* to conspecific hemolymph when in their field-collected shells. None of the behavioral parameters showed a significant difference between test and control periods (t test values associated with $P > 0.05$). Following the clipping of the shell apertures, these crabs did show a strong response to conspecific hemolymph with regards to increases in locomotion ($t = 5.89$, $df = 14$; $P < 0.001$) and in both types of grasping behavior (brief grasps, $t = 2.43$, $df = 14$; $P = 0.029$; long grasps, $t = 3.23$, $df = 14$; $P = 0.006$) (Figure 3). The responses of individuals of *C. infraspinatus* to test solutions prepared from the other species

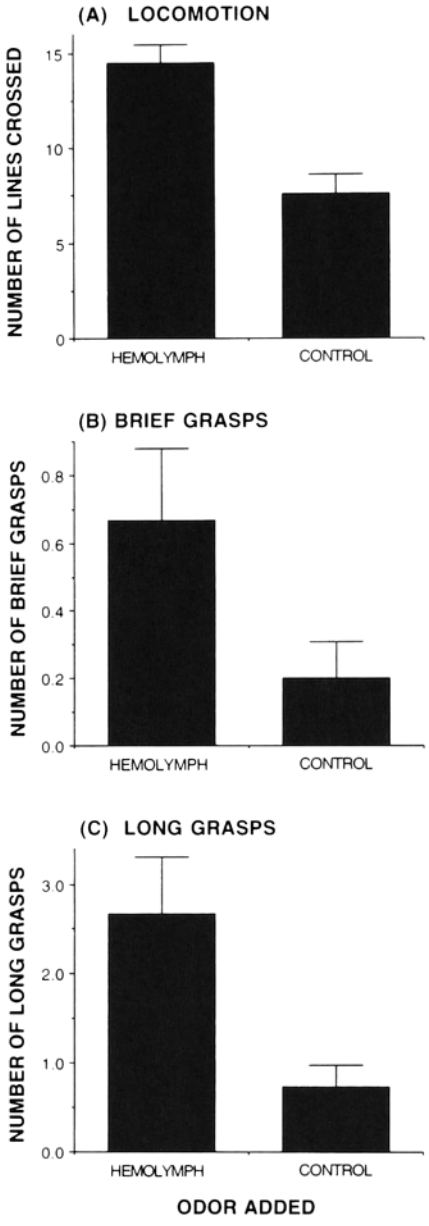

FIG. 3. Behavioral scores (mean + standard error) for individuals of *Clibanarius infraspinatus* during control water and conspecific hemolymph introductions.

TABLE 1. RESULTS OF STATISTICAL TESTS COMPARING BEHAVIORAL RESPONSES OF *Clibanarius infraspinatus* INDIVIDUALS TO CONTROL AND TEST SOLUTIONS.

Source of hemolymph	*df*	Behaviors Recorded					
		Rate of locomotion		Brief grasps		Long grasps	
		t	*P*	*t*	*P*	*t*	*P*
C. infraspinatus	14	5.89	<0.001	2.43	0.029	3.23	0.006
C. taeniatus	14	4.76	<0.001	4.02	0.001	2.88	0.013
C. virescens	15	5.42	<0.001	1.87	0.082	3.37	0.005
Diogenes avarus	19	4.67	<0.001	1.60	0.131	6.57	<0.001

[a]Test solutions were made using the hemolymph from different species of sympatric hermit crabs.

of hermit crabs were also significantly different between test and control periods for every species (Table 1) for at least the rate of locomotion and the number of long grasps [which are the more typical responses shown to hemolymph (Hazlett, 1996a)]. There were no differences among the species as sources of input for individuals of *C. infraspinatus* (Figure 4). Testing the magnitude of the difference between test and control periods, there were no species effects on the magnitude of increase in locomotion (ANOVA $F = 2.20$, $df = 6, 92$; $P = 0.098$), frequency of brief grasps ($F = 1.62$, $df = 6, 92$; $P = 0.195$), or frequency of long grasps ($F = 1.19$, $df = 6, 92$; $P = 0.323$). All of the diogenid species tested elicited responses of similar magnitude, although individual comparisons indicated that the scores for locomotion were significantly different between hemolymph from *C. infraspinatus* and *D. avarus* ($F = 5.93$, $df = 1, 92$; $P = 0.018$).

When individuals of *C. infraspinatus* were stimulated with both snail odor and conspecific hemolymph at the same time, they showed responses characteristic of hemolymph alone (Figure 5). A comparison of the three conditions (snail alone, hemolymph alone, combination) yielded a very significant difference among treatments for change in locomotion ($F = 6.23$, $df = 1, 43$; $P = 0.004$) and change in the frequency of long grasps ($F = 5.94$, $df = 1, 43$; $P = 0.005$). There was no effect on brief grasps ($F = 1.26$, $df = 1, 43$; $P = 0.294$). Individual comparisons showed that for locomotion and long grasps there were significant differences between hemolymph and snail and between the combination stimulus and snail odor (all $P < 0.01$), but no difference between the combination and hemolymph ($P > 0.60$). Both of the odors stimulated grasping and increases in locomotion when presented alone but they did not at all sum together in their effects when presented at the same time.

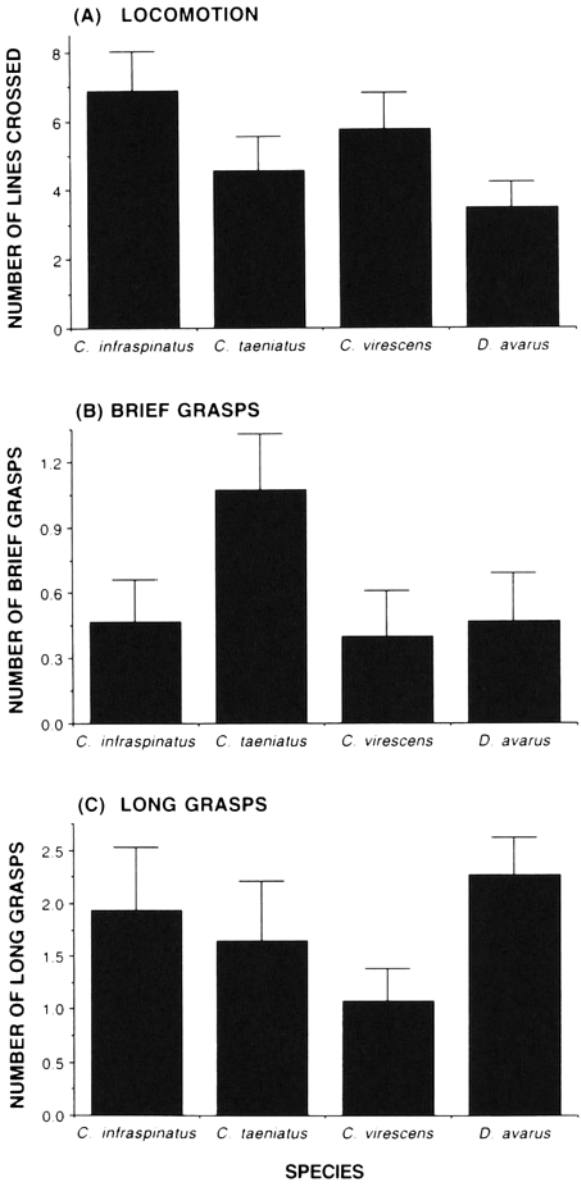

FIG. 4. Difference (mean + standard error) in behavioral scores for individuals of *C. infraspinatus* between control water introductions and introduction of the hemolymph of four species of hermit crabs.

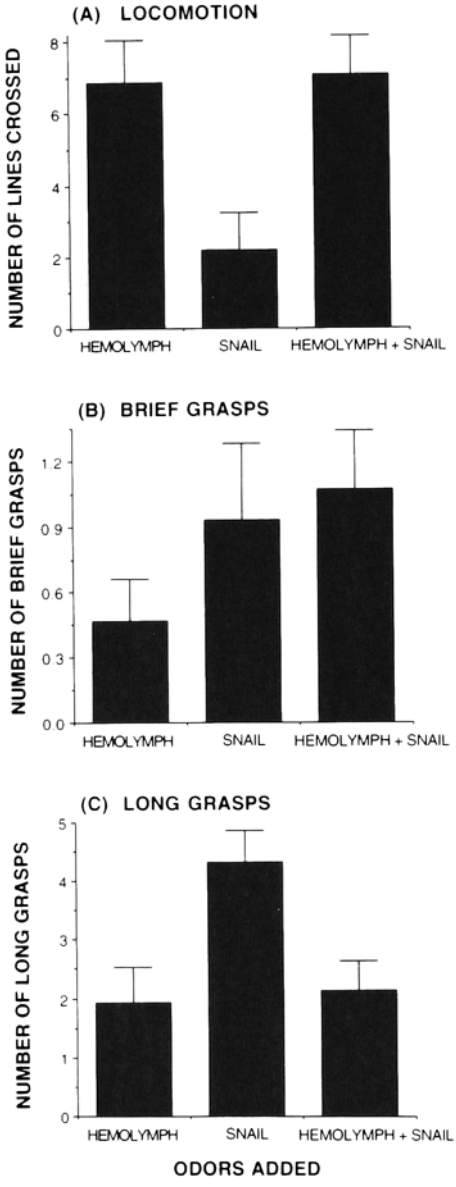

FIG. 5. Difference (mean + standard error) in behavioral scores for individuals of *C. infraspinatus* during control water and test odor introductions.

DISCUSSION

The use by hermit crabs of chemical cues that increase their chances of finding an empty gastropod shell is clearly widespread. In addition to species found along the southeastern coast of the United States, all four of the species tested in Australia showed strong responses to odors from degraded snail flesh. Given the importance of the shell resource to hermit crabs (Hazlett, 1981), the use of an accurate indicator of empty shells is understandable.

While all four Indo-Pacific species showed a significant response to snail odor, the variation in the strength of the response is quite interesting. The lack of an increase in locomotion after detecting snail odor shown by several of the species is puzzling since an increase in locomotion is one of the responses reported for other species (Hazlett, 1996a). The large increase in the number of long grasps induced by snail odor in *C. infraspinatus* and *C. taeniatus*, compared to the more modest (but significant) increase shown by individuals of *C. virescens* and *D. avarus*, is somewhat surprising, because this pattern is different than the responses of *C. vittatus* studied in North Carolina (Hazlett, 1996a), which physically most closely resembles *C. infraspinatus* and *C. taeniatus*. The pattern of a more marked increase in brief grasps shown by *D. avarus* and *C. virescens* is similar to that shown by *C. vittatus*. However, this may reflect something as simple as the generally slower movement of *C. infraspinatus* and *C. taeniatus* as compared to the other species.

The total number of grasps induced by detection of snail odor was significantly weaker for one species (*C. virescens*). This is the only species commonly found in a different habitat, the rocky intertidal zone, compared to the other species tested. *Clibanarius infraspinatus*, *C. taeniatus*, and *Diogenes avarus* (this study), as well as *C. vittatus*, *Pagurus longicrapus*, and *P. pollicaris* (McClean, 1974; Rittschof, 1980a), all inhabit sloping sandy beaches or mudflat areas. These areas would seem to have greater opportunities for orientation to sources of chemical cues due to the more predictable patterns of water movement at low tide as compared to the rocky intertidal zone. Preliminary field observations on *Calcinus tibicen* in the Caribbean and *Calcinus elegans* in Hawaii (both from rocky intertidal areas) also indicated a low level of responsiveness to snail odor (Hazlett, unpublished observations) compared to the species from sandy beaches. An alternative that cannot be ruled out with the data available is that crabs in rocky intertidal areas are in better-fitting shells and thus are less responsive to shell-related odor cues.

The responses of individuals of *Clibanarius infraspinatus* to preparations containing conspecific hemolymph adds to the list of hermit crabs that show a shell-related response to this type of chemical cue. Hermit crabs in poorly fitting shells respond to conspecific hemolymph by grasping nearby gastropod shells (Rittschof et al., 1992) and investigating them, i.e., executing grasps that are

long in duration (Hazlett, 1996a). In this study, the surprising result is the similarity of responses by individuals of *C. infraspinatus* to solutions containing hemolymph from other species. Even though one of the species lives in a different habitat (*C. virescens*) and one is in a distantly related diogenid genus (*Diogenes avarus*), the overall response level to the different solutions was very similar. This is in contrast to the results reported by Rittschof et al. (1992), in which *C. vittatus* did not respond at all to hemolymph from two *Pagurus* species that live in the same area and the responses of the *Pagurus* species were much weaker to hemolymph from other than conspecifics. It is possible this results from greater phylogenetic differences in the case of diogenid and pagurid species since they are in different families. Some have suggested that these two families represent separate evolutions of the hermit crab life-style (McDonald et al., 1957), although McLaughlin (1983) has argued for monophyly. This possible explanation can also apply to the results of Thacker (1994) on terrestrial species where *Coenobita compressus* (Family Coenobitidae) responded just as strongly to conspecific hemolymph odors and odors from the hemolymph of the congeneric species *Coenobita clypeatus*, but the land hermit crabs did not respond to the hemolymph of the aquatic diogenid species he tested. Since hermit crabs are rarely specialists on any particular shell type (Hazlett, 1981), it seems reasonable that any injured hermit crab is a potential source of an empty shell for almost any other hermit crab, irrespective of the species of crab.

The response of individuals of *C. infraspinatus* to the combination of hemolymph odor and snail odor are very similar to the responses shown by individuals of *C. vittatus* when presented with a combination of inputs (Hazlett, 1996a; Rittschof and Hazlett, unpublished data). In both species, hemolymph-related responses seemed to overrule any snail-induced responses. While shell-seeking responses to detection of conspecific (or other hermit crab) hemolymph are shown only by crabs in poorly fitting shells (Rittschof et al., 1992), those crabs appear to be more strongly influenced by that cue than by the more specific cue resulting from degradation of snail flesh. While it is possible that in both studies reporting this result, the concentration of the hemolymph cue was stronger than the concentration of the snail cue, there is an alternative explanation. The hemolymph cue, while indicating some degree of danger, is a more general signal of shell availability since the potentially empty shell can be any species of shell in the area, while the snail flesh cue provides information on the potential availability of a single species of shell. While detection of one cue frequently inhibits the responses associated with another cue, the hierarchy of influence is usually one of predator cues overruling resource-related cues (Hazlett, 1996a,b; Mitchell and Hazlett, 1996). In this study, the inhibition is by the more reliable of the resource-related cues.

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3-METHYL-α-HIMACHALENE: PROPOSED STRUCTURE FOR NOVEL HOMOSESQUITERPENE SEX PHEROMONE OF *Lutzomyia longipalpis* (DIPTERA: PSYCHODIDAE) FROM JACOBINA, BRAZIL

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Abstract—The principal behaviorally active volatile component (ca. 90%+) of the sex pheromone glands of *Lutzomyia longipalpis* from Jacobina, Brazil, has been isolated and characterized as a novel homosesquiterpene with the structure 3-methyl-α-himachalene ($C_{16}H_{26}$). A minor component (ca. 10%) of the gland extract has also been identified as the sesquiterpene α-himachalene ($C_{15}H_{24}$). This work confirms that there are at least 3 members of the *L. longipalpis* species complex.

Key Words—Sex pheromone, tergal gland, *Lutzomyia longipalpis*, 3-methyl-α-himachalene, homosesquiterpene, mass spectrometry, high-performance liquid chromatography, nuclear magnetic resonance.

INTRODUCTION

The sandfly, *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae) is the vector of the protozoan parasite *Leishmania chagasi* (Cunha & Chagas) (Kinetoplastida: Trypanosomatidae), the causative agent of visceral leishmaniasis in the New World. Male *L. longipalpis* release a sex pheromone from glands that can be seen as pale patches on the third or third and fourth tergites of the abdomen (Lane et al., 1985). The pheromone can attract females (Ward

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et al., 1989; Hamilton et al., 1994) from distances of up to 60 cm to filter paper discs that have been impregnated with extracts prepared from whole pheromone glands (Morton and Ward, 1989).

The sex pheromone gland of *L. longipalpis* from Jacobina in northeastern Brazil contains several detectable compounds. However, only the principal volatile component of the gland extract (90%+) is responsible for the attraction of females. In bioassays performed with isolated gland components, the second most abundant compound (ca. 10%) and other trace compounds did not influence the activity of the sex pheromone (Hamilton et al., 1994). Further evidence that only the major component of the gland extract was pheromonally active was provided by Dougherty et al., (1995), who showed that only one type of olfactory cell in the ascoid sensillum on the female antenna was stimulated by the sex pheromone gland extract.

L. longipalpis is a species complex. Gas chromatography–mass spectrometry analysis by Lane et al., (1985) and Phillips et al., (1986) identified two populations based on unidentified components produced by the pheromone glands. One population produced compounds with a molecular weight of 218 and the other a molecular weight of 272. These molecular weights and the fragmentation patterns suggested that the components were homosesquiterpene ($C_{16}H_{26}$) and diterpene ($C_{20}H_{32}$) hydrocarbons. Hamilton and Ward (1991) suggested that there could be up to six different populations of *L. longipalpis* and that the homosesquiterpene-producing populations could be further subdivided on the basis of the type of homosesquiterpene they produced. Recently, a new homosesquiterpene has been found in a population of *L. longipalpis* from Costa Rica (Hamilton et al., 1996b) and a diterpene has been found in *L. pessoai*, a related species (Hamilton and Ward, 1994).

The sex pheromone of *L. longipalpis* from Lapinha in southern Brazil has recently been characterized as a novel homosesquiterpene, with the structure proposed as 9-methylgermacrene-B (Hamilton et al., 1996a). The objective of this current work was to characterize the homosesquiterpene pheromone of the Jacobina population of *L. longipalpis*.

METHODS AND MATERIALS

Insects. L. longipalpis were maintained in colony as described previously by Hamilton et al., (1994) at a temperature of 26°C, 98% relative humidity, and a 12 L:12 D photoperiod. The colony was established from adults collected in Jacobina, Bahia State, Brazil (40°31'W 11°11'S). Extracts for analysis were prepared either from dissected glands or by extracting large numbers of whole male flies in hexane (Hamilton et al., 1994, 1996a). Extracts prepared from whole sandflies were filtered through glass wool to remove solid debris prior to

further analysis and preparative HPLC. All extracts were reduced under N_2 to produce an extract with a final concentration of one male equivalent (ME) per microliter.

Column Chromatography. Column chromatography was performed on a silica gel micro column prepared by placing BioSil-A (20–44 μm) (BioRad, Hemel Hempstead, Hertfordshire, UK) silica gel (0.25 g) in a Pasteur pipet. The column was precleaned by eluting sequentially with hexane (10 ml) (pesticide grade, BDH, Poole, Dorset, UK) followed by diethyl ether (10 ml) (chromatography grade, BDH). The hydrocarbon fraction was eluted by hexane (3 column volumes).

Gas Chromatography. GC analysis was carried out using a J&W DB-1 capillary column (Alltech, Carnforth, Lancashire, UK) (30 m × 0.32 mm ID, 0.25-μm phase thickness) in a Shimadzu model 15-A gas-chromatograph (FID 310°C) (Shimadzu Corp. Kyoto, Japan) linked to a Shimadzu CR-5A data-pac integrator. Injection of extract was via a Grob split/splitless injector in the splitless mode (0.6 min sampling time) at 180°C. Carrier gas was helium at 1 ml/min. The GC was temperature programmed with an initial 2 min at 45°C, then a rise of 10°C/min to 250°C followed by a rise of 15°C/min to a final isothermal period at 310°C (4 min). The detector (flame ionization) temperature was set at 310°C.

High-Pressure Liquid Chromatography (HPLC). Analysis was carried out on two Waters Resolve C18 analytical columns (3.9 × 300 mm) in series with a Resolve C18 precolumn, heated to 30°C in a Waters HPLC; 600E multisolvent pump and gradient controller, U6K injector, and 991 photodiode array detector (Waters, Watford, UK). The mobile phase was acetonitrile–isopropanol (70:30) at 1 ml/min, sparged with helium at 25 ml/min prior to and during analysis. Standards used for calibration and monitoring of separations were (−)-β-caryophyllene and caryophyllene oxide (Aldrich, Gillingham, UK) (1 μg/μl, 10 μl injection). Fractions for further GC, GC-MS, and NMR analysis were collected manually in 10-ml acid-cleaned beakers as they eluted from the HPLC detector. The nonpolar fraction of the gland extract was recovered from the relatively polar mobile phase by a liquid–liquid separation.

Gas Chromatography–Mass Spectrometry (GC-MS). Coupled gas chromatography–mass spectrometry was performed on a Hewlett-Packard 5880A GC with an HP-1 capillary column, 50 m × 0.32 mm ID, 0.25-μm film thickness, directly coupled to a VG 70-250 MS, electron impact (EI), 70 eV, 250°C. Sample was introduced via a cold on-column technique. The GC was temperature programmed with an initial 5 min at 30°C, then a rise of 5°C/min to a final isothermal period at 250°C.

Additional analysis was carried out on a Hewlett Packard 5890 II+ gas chromatograph with an HP-5MS capillary column, 30 m × 0.25 mm ID, 0.25-μm film thickness, directly coupled to a Hewlett Packard 5972A benchtop mass

spectrometer, EI, 70 eV, 165°C. Samples were introduced via a split/splitless injector (splitless mode, sampling time 0.7 min, 180°C). The GC was temperature programmed with an initial 2 min at 50°C, with an increase of 10°C/min to a final isothermal period at 250°C (10 min).

GC-MS studies were performed on crude extracts from dissected glands, hexane fractions from the BioSil-A column, and on reduction and epoxidation products.

Hydrogenation of Sex Pheromone. Ten male equivalents of *L. longipalpis* extract (10 μl) were placed in a small micro reaction vessel prepared from a Pasteur pipet. Palladium black (20 μg) (Aldrich Chem. Co.) catalytic reducing agent was added and the mixture was shaken. Hydrogen was blown over the solution for 2 min. The micro vial was inverted, trapping hydrogen in the bulb of the vial, and then placed (inverted) in a 25-ml inverted screw-top glass tube that was filled with hydrogen, sealed, and kept at room temperature for 2 hr prior to analysis.

Epoxidation. Dimethyldioxirane (DMD) reagent was prepared as described by Adam et al. (1987). *L. longipalpis* extract (100 μl = 100 ME) was eluted from a Florisil (350 mg) pipet column in hexane (2 ml). Volume was reduced under N_2 to 100 μl and placed in a 1-ml vial and reagent (40 μl) added. The reaction was allowed to proceed for 24 hr before analysis by GC and GC-MS.

Standards. α-Himachalene was obtained from the nonpolar fraction of the Himalayan deodar (*Cedrus deodara*, Loud.) (Krishna Rao et al., 1952). A series of *n*-alkane standards, C_{12}–C_{21} (Sigma Chem. Co., Poole, UK) were analyzed by GC to provide comparative retention time data.

NMR. HPLC collected material was prepared for NMR by partitioning the HPLC eluent in double-distilled H_2O and distilled ether. The organic phase was reduced under N_2 and gradually exchanged with deuterochloroform ($CDCl_3$).

NMR spectra were recorded in $CDCl_3$ solution using tetramethylsilane as the interal standard (δ = 0.0). Proton NMR spectra (^{1}H NMR) were recorded at 400 MHz on a Jeol JNM-GX400 with Fourier transform facility. The ^{13}C NMR spectra were similarly recorded at 100 MHz.

RESULTS

GC, HPLC, and Silica Gel Column. Analysis of crude extract by GC gave a small, sharp peak at 9.90 min (11%) (compound a) and a large, sharp peak at 10.47 min (89%) (compound b) (the sex pheromone, Hamilton *et al.*, 1994). A number of other compounds were present in trace amounts between 8.8 min and 12.07 min. The hexane eluant of the silica gel column gave both compounds, a and b, on GC. HPLC of the crude *L. longipalpis* extract gave a small peak at 10.62 min (10.6%) (compound c, λ_{max} 202.8 nm) followed by one large

peak at 10.92 min (89.3%) (compound d, λ_{max} 204.2 nm). These HPLC peaks were resolved on the baseline, and when collected, back extracted, and analyzed by GC, were found to be equivalent to GC compounds a and b, respectively.

Gas Chromatography–Mass Spectrometry (GC-MS). The mass spectrum of compound b (R_t = 38.05 min) is given in Figure 1. The mass spectrum of compound a (R_t = 36.73 min) was found to be *m/z* 204 (M^+, 35), 93(100), 94(81), 41(78), 119(72), 79(64), 69(59), 105(57), 90(49), 121(44), 161(42), 189(42), 55(42), 131(40), 147(18), 175(6).

Upon injection of the reduction products of the whole extract, two main isomer peaks were found with similar spectra: R_t = 38.22 min, *m/z* 222 (M^+, 6), 55(100), 70(96), 41(83), 97(62), 81(54), 123(53), 109(21), 151(17), 137(10), 207(9), and R_t = 38.90 min, *m/z* 222 (M^+, 6), 100(55), 69(85), 41(81), 81(60), 97(60), 123(58), 109(22), 151(16), 137(12), 207(8).

Analysis of the epoxidation products of the whole extract gave two principal isomeric products: R_t = 15.63 min, *m/z* 250 (M^+, 10), 73(100), 60(89), 43(82), 129(34), 185(22), 83(20), 109(16), 97(15), 228(12); and R_t = 16.75 min, *m/z* 250(M^+, 18), 43(100), 100(80), 55(68), 178(40), 69(33), 81(23), 109(23), 135(23).

NMR. Proton ^1H and 2D ^1H–^1H NMR experiments were done on isolated sex pheromone, compound b. Full ^1H NMR assignments were not possible in the 1.1- to 2.1-ppm region due to the complexity of the spectrum and some impurities, but clear signals were obtained at 0.97(s), 1.01(s), 1.02 (d, J = 7), 2.30(m), 2.81(m), 4.77(s), 4.82(s), 5.52(m). For ^1H–^1H NMR, strong corre-

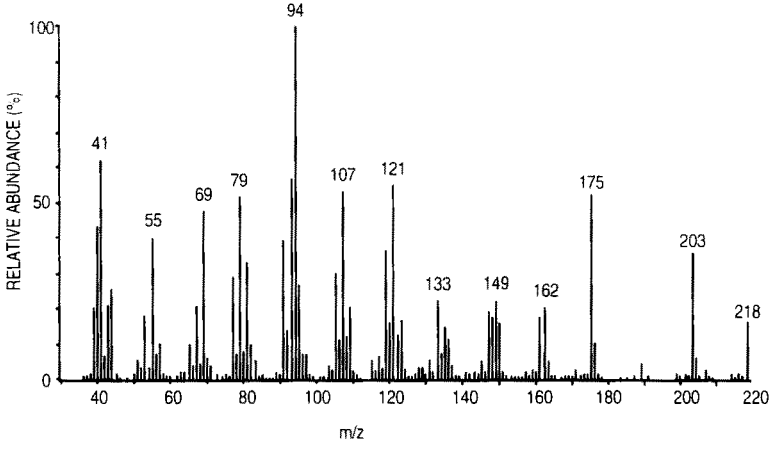


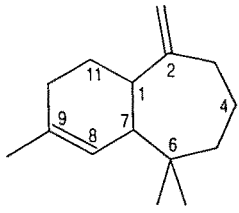
FIG. 1. Mass spectrum of homohimachalene from *Lutzomyia longipalpis* from the Jacobina region of Brazil. (Compound b)

lations were observed between shifts at: 5.52–1.65, 2.81–2.10, and 2.30–1.02 ppm. Full NMR assignment of the α-himachalene standard was achieved using 2D NMR experiments (^{1}H-^{1}H and ^{13}C-^{1}H correlations (Figure 2). The strong ^{1}H-^{1}H correlations are shown in Figure 2B with arrows.

DISCUSSION

HPLC analysis and elution from a Florisil column by hexane suggested hydrocarbon structures for a and b. The mass spectra suggested a molecular weight of 204 amu for compound a and 218 amu for compound b. Reduction and epoxidation of b gave products with molecular weights higher by 4 amu (222, m^+) and 32 amu (250, M^+), respectively, establishing the presence of two double bonds. UV λ_{max} data for c (= a) and d (= b) indicated that these double bonds were not conjugated.

GC-MS analysis of the components a and b of the pheromone glands of *L. longipalpis* from Jacobina suggested a sesquiterpene as the minor component and a homosesquiterpene as the later-eluting major component. The sesquiterpene (a) had a spectrum closely similar to that published for α-himachalene (NIST, 1990). α-Himachalene from the essential oil of Atlas cedarwood, authenticated by MS and NMR spectroscopy, gave peak enhancement on GC with the *L. longipalpis*-derived product. This confirmed the minor component (compound a) as α-himachalene (structure I). Although this did not confirm the absolute



I

stereochemistry, the *cis* configuration is assumed for compound a because of identity with authentic α-himachalene for which the *cis* configuration has been proven (Tabacchi et al., 1974).

The proposed homosesquiterpene (compound b) had a mass spectrum similar to that expected for an α-himachalene but an extra methyl group was indicated. There were also strong similarities between the proton NMR spectra of α-himachalene and the sex pheromone compound b. The *gem*-dimethyl groups gave absorbances at 0.97(s) and 1.00(s) for α-himachalene compared with 0.97(s) and 1.01(s) for compound b. The protons on the bridgehead C-1 in α-him-

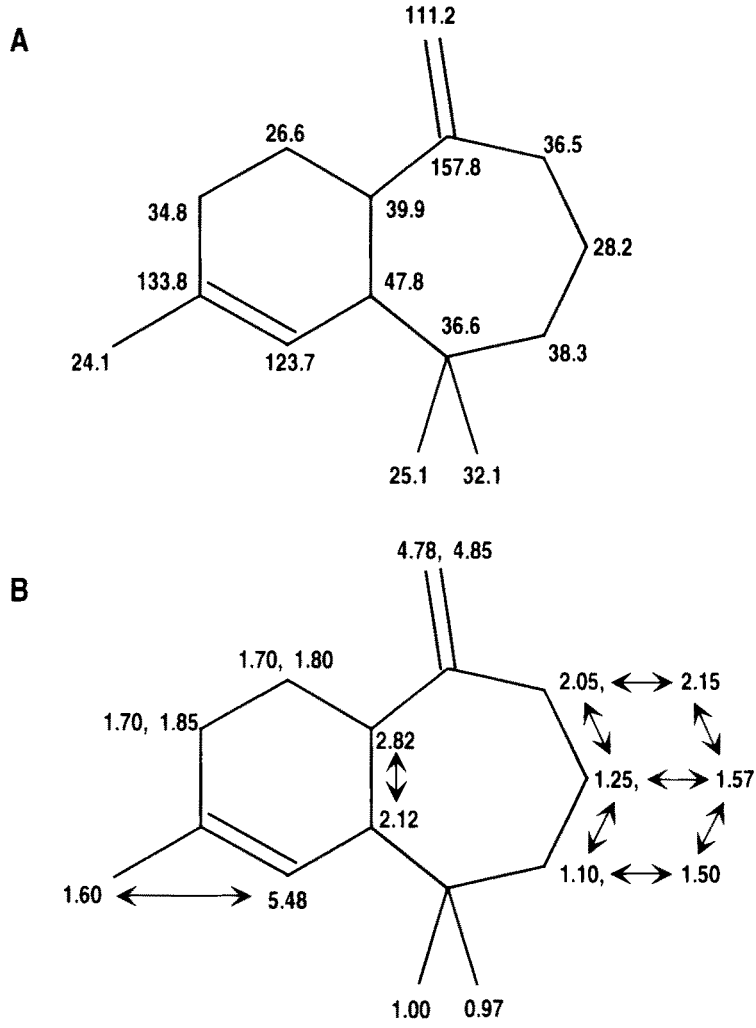


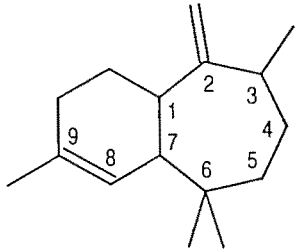
FIG. 2. NMR assignments for α-himachalene compound a: (A) ^{13}C assignments, (B) ^{1}H assignments (strong ^{1}H–^{1}H correlations are shown with arrows). ^{1}H–^{13}C correlations are consistent with these assignments.

achalene gave an absorbance at 2.82 compared with 2.81 ppm in compound b. The exomethylene at C-2 in α-himachalene gave absorbancies at 4.78 and 4.85 compared with 4.77 and 4.82 ppm in compound b. The C-8 olefin in α-himachalene gave an absorbance at 5.48 compared with 5.52 ppm in compound b.

As with α-himachalene, compound b showed strong correlations between the methyl on C-9 (1.65) to olefinic proton on C-8 (5.52), and also between the bridgehead protons on C-1 and C-7 (2.81 and 2.10). These spectral details strongly support the α-himachalene type structure for the pheromone.

The pheromone has an additional methyl group at 1.02 ppm as a doublet with the 7-Hz coupling to a proton at 2.30 ppm. The abolition of strong ^1H-^1H correlations in the pheromone for the protons on carbons 3, 4, and 5 (Figure 2B) suggests positioning of the methyl in this part of the cycloheptane ring. From biosynthetic considerations, C-3 is the favored position (Hamilton et al.,1996a) i.e., the homohimachalene equivalent to the 9-methylgermacrene proposed as comprising the sex phermone of another member of the *L. longipalpis* species complex from Lapinha (Minas Gerais State, Brazil).

Thus, the proposed structure for the sex pheromone is 3-methyl-α-himachalene, systematically named 2-methylene-3,6,6,9-tetramethylbicyclo-[5,4,0]undec-8-ene (structure II).



II

The two isomeric GC peaks for the reduced and the epoxidized products presumably arose from the diastereoisomers generated by the asymmetry at C-2 and C-9 in these products. The change in the base peak from *m/z* 93 to 94 caused by the additional methyl group (although *m/z* 94 and 93 remain abundant) is not yet explained, but a rearrangement of the *exo*-double bond in the MS source could in turn allow generation of a monocyclic C_7H_{10} radical ion (*m/z* 94). Synthesis of the candidate compound with structure II is now in progress to confirm this assignment and to establish the absolute stereochemistry of the molecule at C-1, C-3, and C-7. Comparison with authentic α-himachalene suggests that the C-1 and C-7 positions of compound b (structure II) have the *cis* configuration. Insufficient compound b was available for further structural confirmation by more sophisticated NMR experiments.

Sex pheromones are responsible for mate discrimination (Roelofs and Comeau, 1969) and as such can function in species isolation. Characterization of sex pheromones can be a valuable aid in the differentiation and classification of species where no morphological differences are discernible (Roelofs and Cardé, 1974; Dugdale, 1990; Löfstedt et al., 1994). Within the *L. longipalpis*

species complex, sex pheromones are indicative of the reproductively isolated groups of sandflies first described by Ward et al. (1983, 1988) and, along with other prezygotic mating barriers such as interpulse sound frequencies produced by wing beats (Ward et al., 1988) and postzygotic barriers described by Lanzaro et al. (1993), maintain species isolation in sympatric populations. This work has shown that in the *L. longipalpis* species complex there are at least two populations (taxa) producing different homosesquiterpene sex pheromones.

The 9-methylgermacrene-B structure previously proposed for the sex pheromone of *L. longipalpis* from Lapinha (Hamilton et al., 1996b) and 3-methyl-α-himachalene could be derived from a common precursor such as 8-methylfarnesylpyrophosphate, although the alternative methyl migration or methylation cannot be ruled out. The biosynthetic relationship of these compounds with the diterpene produced by some members of the species complex is unclear. Recent work has shown that in the Sobral region of Brazil some members of the complex product diterpene exclusively, whereas other members of the complex produce predominantly 9-methylgermacrene-B with small amounts of diterpene (Hamilton and Ward, unpublished observations). There is the possibility that the homosesquiterpenes arise through modification of a common diterpene precursor (by, for example, oxidative loss of four carbons) analogous to biosynthesis of the homoterpenes (Boland and Gäbler, 1989; Donath and Boland, 1994). Nonetheless the homosesquiterpene structures currently proposed indicate a structure for a diterpene precursor unexpected from normal isoprenoid biosynthesis.

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QUANTIFICATION OF CONTACT OVIPOSITION STIMULANTS FOR BLACK SWALLOWTAIL BUTTERFLY, *Papilio polyxenes*, ON THE LEAF SURFACES OF WILD CARROT, *Daucus carota*

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Abstract—Ovipositing black swallowtail butterflies, *Papilio polyxenes*, make their final host-selection decisions on the basis of compounds present on the leaf surface. Little information is available, however, on the chemistry of leaf surfaces. The purpose of this study was to develop a technique to extract and quantify the concentrations of compounds from the leaf surfaces of *Daucus carota*, one of the main host species for *P. polyxenes*, with particular reference to compounds already identified as contact oviposition stimulants, namely *trans*-chlorogenic acid (CA) and luteolin-7-*O*-(6″-*O*-malonyl)-β-D-glucopyranoside (L7MG), as well as its degradation product luteolin-7-glucoside (L7G). Plant surfaces were extracted by dipping leaves sequentially in pairs of solvents: (1) $CHCl_3$–MeOH, (2) near-boiling H_2O, (3) $CHCl_3$–near-boiling H_2O, and (4) CH_2Cl_2–CH_2Cl_2. The resulting extracts were fractionated and analyzed using high-performance liquid chromatography. The leaf-surface concentrations of each compound were calculated using regressions relating leaf surface area to leaf weight that were obtained from measurements of field-collected carrot plants. All four methods removed the three compounds from carrot leaf surfaces, but the solvent systems differed in effectiveness. The chloroform–near-boiling water solvent system performed better than the other solvent combinations, but not significantly so. This system also extracted the highest number of polar, UV-absorbing compounds. Methylene chloride was significantly less efficient than the other methods. An additional test confirmed

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that the chloroform–near-boiling water method removed compounds from the surface alone and probably not from the apoplast or symplast. Surface concentrations of CA (up to 600 ng/cm^2 leaf surface) were substantially greater than those of the two flavonoid compounds. No clear seasonal trend in concentrations was evident from the limited number of sampling dates.

Key Words—*Papilio polyxenes*, Papilionidae, *Daucus carota*, Apiaceae, oviposition, leaf surface, contact chemoreception, HPLC, flavonoid glycosides, chlorogenic acid.

INTRODUCTION

The initial host plants of the larval offspring of the black swallowtail butterfly, *Papilio polyxenes*, are chosen by the egg-laying female adult (Blau, 1981; Feeny et al., 1983, 1985). A gravid female locates a potential host plant on the basis of olfactory and visual cues, which may lead the female to land on the plant (Feeny et al., 1989). Once on a leaf, the female chooses whether or not to oviposit after sensing chemical cues on the leaf. Upon landing, the female drums her foretarsi on the leaf surface, bringing contact chemoreceptors located on the ventral surface of the foretarsi into contact with chemical stimuli present on the leaf surface (Feeny et al., 1983; Roessingh et al., 1991). Based on the stimuli perceived through this drumming behavior, the *P. polyxenes* female may curl her abdomen and oviposit. As in several other groups of phytophagous insects (Städler, 1986; Traynier and Hines, 1987; Linley, 1989), contact chemoreception during oviposition by butterflies appears to be limited to the leaf surface itself; no studies have yet revealed any penetration of the leaf cuticle due to drumming behavior (e.g., Städler, 1986; Bart and Williams, 1993; but see Boppré, 1978, 1983 for exception in Danaidae).

Larvae of *P. polyxenes* feed almost exclusively on plants of the family Apiaceae (Tietz, 1972). In New York, the main host plant of *P. polyxenes* is the umbellifer *Daucus carota* (Feeny et al., 1985). Commonly known as wild carrot or Queen Anne's lace, this plant is a winter annual found throughout the northeastern and northcentral United States where it has a patchy distribution and occurs in secondary successional areas (Peterson and Simon, 1986). From ethanolic extracts of carrot foliage, Feeny et al. (1988) identified two major contact stimulants for *P. polyxenes*: *trans*-chlorogenic acid (CA) and luteolin-7-*O*-(6″-*O*-malonyl)-β-D-glucopyranoside (L7MG), a flavonoid malonate. Roessingh et al. (1991) confirmed the stimulatory characteristics of these compounds and identified some of the chemoreceptors in *P. polyxenes* that respond to these stimuli. Feeny et al. (1988) discovered that the flavonoid malonate was an unstable compound that degraded under natural conditions when the malonic acid group dissociated from the flavonoid. Luteolin-7-glucoside (L7G), the fla-

vonoid hydrolysis product, was also recovered from ethanolic extracts of carrot foliage. It was not an oviposition stimulant for *P. polyxenes*, and preliminary evidence suggested it may be an oviposition deterrent. Additional stimulant compounds, as yet unidentified, were present in the carrot fractions containing bases and polar neutrals (sugars and inositols) (P. Feeny et al., unpublished data).

Past work on the identification of the oviposition stimulants for *P. polyxenes* from *D. carota* has been based on whole-leaf extracts. Since stimulant isolation and chemical characterization require large quantities of plant material, whole-leaf extracts are necessary in order to obtain sufficient amounts of the compounds of interest. It has been assumed, but never proved, that the stimulant compounds are present on the leaf surface (Feeny et al., 1983).

Although Richmond and Martin (1959) reported the extraction of phenolic compounds from apple leaves by 10-sec dips in diethyl ether, they noted some leaching of compounds from the leaf interior, and it remains unclear how much material originated from the leaf surface. There has been much subsequent discussion as to the likelihood that flavonoid glycosides and other polar compounds can occur within the nonpolar waxy matrix of the leaf surface (Harborne, 1973; Hunt and Baker, 1980; Wagner, 1982; Robinson, 1991). Recent studies, reviewed below, have demonstrated clearly that several classes of polar compounds are indeed present on leaf surfaces, although the mechanisms of transport and storage remain unclear.

Oviposition stimulants for insects have typically been extracted from leaf surfaces by dipping leaves for several seconds in various solvents. The suitability of different solvents for particular extractions depends critically on the polarity of the stimulant compounds. Most chemical explorations of the leaf surface have used methylene chloride to extract the surface compounds (Städler and Buser, 1984; Proksch et al., 1985, 1986; Bohm and Constant, 1990; Bohm and Chan, 1992; Bohm, 1993; Reid and Bohm, 1993). This organic solvent of medium polarity has been used with success to extract sesquiterpenoids and other relatively nonpolar compounds (Städler and Roessingh, 1991), but it may have been insufficient to remove polar compounds present in the epicuticular waxes or the cuticle proper. Cold water was used to extract more polar compounds such as fructose and other water-soluble carbohydrates from leaf surfaces of *Zea mays* (Derridj et al., 1987; Fiala et al., 1990). Neither cold water nor methanol alone were effective in extracting stimulants for *Delia radicum* or *Pieris rapae*; however, extraction of glucosinolates and other stimulants for these insects required either hot water or methanol preceded by a chloroform or methylene chloride dip (Städler and Roessingh, 1991; Renwick, et al., 1992; van Loon et al., 1992; Baur et al., 1996). Hot water was also used by Zobel and Brown (1988) to extract furanocoumarins from the leaves of *Ruta graveolens*. Städler and Roes-

singh (1991) concluded that stimulants must generally be embedded quite deeply in the waxy leaf cuticle so that they can be extracted only when the waxy layer is dissolved (chloroform/methylene chloride) or melted (hot water).

In early work on oviposition stimulants for *P. polyxenes*, Feeny et al. (1983) found that stimulant activity could not be extracted from the surfaces of carrot leaves by individual organic solvents or by cold water. In fact, the leaves retained their stimulant activity even after immersion in 2 N HCl for 30 min at 40°C; only as temperatures were increased above 60°C, presumably causing softening and disruption of the waves, did the leaves lose their ability to stimulate oviposition. Since the major stimulants, CA and L7MG, are both easily hydrolyzed by dilute HCl, this was evidence that these polar stimulants are indeed embedded in the surface wax, as concluded by Städler and Roessingh (1991).

The purpose of this study was threefold: (1) to confirm the presence of contact oviposition stimulants for *P. polyxenes* on the leaf surfaces of *D. carota*, (2) to develop the most efficient extraction technique for the removal of these stimulants from the surface of *D. carota* leaves, and (3) to quantify the concentrations of these chemicals on the leaf surface.

METHODS AND MATERIALS

Surface Area Regressions. Foliage of carrot, *Daucus carota*, was taken fresh from the field in the vicinity of Ithaca, New York, in spring 1990 and transported to the laboratory on ice. Additional carrot foliage was taken from greenhouse cultures at Cornell University. All petioles were cut just below the point of attachment of the first leaflets, and the fresh weight and length of each leaf was recorded. All parts of each leaf were then mounted on paper sprayed with Scotch brand Spray Mount Artist's Adhesive (3M). This paper was covered with clear plastic and photocopied to make a permanent record of leaf surface area before wilting began.

The photocopied images of leaves were scanned using an Apple flatbed scanner. On all photocopied sheets, a 0.5-square-inch standard was included for calibration. The pixels of the scanned images were then counted using a pixel-counting program provided by M. Lapidus. The calculated surface area of each leaf was doubled to account for both the upper and lower leaf surfaces. Least-squares regressions were calculated for leaf surface area versus leaf weight and for leaf surface area versus leaf length.

Plant Material for Surface Extracts. Four collections of *D. carota* foliage were taken fresh from the field in the vicinity of Ithaca, New York, from June 1994 to October 1994. The cut petioles of individual leaves were placed in Aquapiks (Cleveland Plant and Flower Co., Wholesale Florists, Binghamton,

New York 13905) and transported to the laboratory on ice. The leaves within a single collection were assigned randomly to four treatment groups. Within each treatment group the fresh weight of the leaves, including petioles, was obtained prior to surface extraction. Each treatment group contained between 10.0 and 13.0 g of leaves. After surface extraction, the petioles were cut from the leaves below the point of attachment of the first leaflets and weighed separately. The petiolar weight was subtracted from the total leaf weight to obtain the laminar weight.

Solvent Extraction Systems. Four solvent systems (treatments) were used to extract chemicals from the surfaces of carrot leaves. For system 1 ($CHCl_3$–MeOH), a freshly cut leaf in this treatment group was dipped in 200 ml of $CHCl_3$ for 10 sec and held above the solvent to drip for 10 sec. The leaf was then dipped in 200 ml MeOH for 7 sec and allowed to drip for 10 sec. All leaves in the group were dipped individually in the same aliquot of solvent. For system 2 (near-boiling H_2O), leaves were dipped individually in 300–350 ml of near-boiling H_2O (HPLC grade, temperature at least 94°C) for 2 sec and were allowed to drip for 10 sec. For system 3 ($CHCl_3$–near-boiling H_2O), each leaf was first dipped for 10 sec in 200 ml of $CHCl_3$ and allowed to drip for 10 sec. The leaf was then dipped for 1 sec in 300 ml of near-boiling H_2O and allowed to drip for 10 sec. For system 4 (CH_2Cl_2–CH_2Cl_2), leaves were dipped individually in 300 ml of CH_2Cl_2 for 30 sec and allowed to drip for 10 sec. This was repeated in a second volume of CH_2Cl_2. Since the CH_2Cl_2 evaporated rapidly, solvent volume was kept constant throughout the extraction by addition of more solvent. All solvent fractions were stored in the freezer until preparation for HPLC analysis.

Solvent Timing Study. Two collections of *D. carota* foliage were taken fresh from the field in the vicinity of Ithaca, New York, in July 1995. The cut petioles of individual leaves were placed in Aquapiks and transported to the laboratory on ice. The leaves from both collections were combined and assigned randomly to six treatment groups. Within each treatment group the fresh weight of the leaves, including petioles, was obtained prior to surface extraction. After surface extraction, the petioles were cut from the leaves below the point of attachment of the first leaflets and weighed separately. The petiolar weight was subtracted from the total leaf weight to obtain the laminar weight. The leaves from each treatment were extracted with solvent system 3 ($CHCl_3$–near-boiling H_2O). In these extractions, the dipping time in $CHCl_3$ was kept constant at 10 sec, but each treatment group received successively longer dipping times in the near-boiling H_2O (with times of 1, 2, 3, 4, 5, and 7 sec, respectively). All solvent fractions were stored in the freezer until preparation for HPLC analysis.

Preparation of Surface Extracts for HPLC Analysis. The organic fractions were concentrated in vacuo at 40°C to a volume of 20–40 ml and then extracted three times with equal volumes of H_2O (HPLC grade). The resulting aqueous

layer was kept, and the original organic layer discarded. The aqueous surface washes were concentrated in vacuo at 40°C to a volume of 20–40 ml and then extracted three times with equal volumes of $CHCl_3$. The original aqueous layer was kept, and the $CHCl_3$ wash discarded. In a solvent system containing both an organic and aqueous wash, the final aqueous layers after extraction for both washes were combined. All final extracts were concentrated in vacuo at 40°C to a final volume of 1 ml.

HPLC Methodology. Analyses of surface extracts were carried out on a Waters multisolvent delivery system (M6000 pump, U6K injector) and monitored between 200 and 600 nm (Waters 991 photodiode array (PDA) detector). The analytical reverse-phase C-18 column (Phenomenex IB-sil, 4.6 × 250 mm, 5 μm with 4.6 × 30 mm C-18 guard column) was eluted at a flow rate of 1.0 ml/min using a stepped gradient (Table 1). Calibration and quantification of the PDA data were done by analysis of eight standard solutions containing CA, L7G, and L7MG (500, 250, 150, 60, 30, 15, 7.5, and 4.875 μg of each compound per ml, respectively, giving from 5000 to 48.750 ng of each compound per injection). Calculation of calibration curves and quantification of the known compounds in the surface extracts was performed by Millennium 2010 Chromatography Manager software (Millipore, Inc.).

Sources of Standard Compounds. CA was purchased from Sigma. L7G was purchased from Indofine Chemical Company. The L7MG standard was purified from fresh *D. carota* foliage using a modification of the procedure described by Feeny et al. (1988). Foliage of carrot was taken fresh from the field in the vicinity of Ithaca, New York, in May 1994. The cut petioles of individual leaves were placed in Aquapiks and were transported to the laboratory in coolers containing ice packs. Leaves, 40 g fresh weight at a time, were

TABLE 1. HPLC ELUTION PROGRAM FOR CHEMICAL ANALYSES OF *Daucus carota* SURFACE EXTRACTS ON ANALYTICAL REVERSE-PHASE C-18 COLUMN[a]

Time (min)	Proportion of 1% aq HOAc	Proportion of MeOH	Curve
0.0	100	0	
7.5	65	35	6
25.0	55	45	9
50.0	55	45	6
55.0	0	100	6
65.0	0	100	6

[a]Curve specifies the rate of change of eluent composition over time (6 produces a linear gradient curve; 9 represents a concave gradient curve).

immediately blended in boiling 95% EtOH for 5–10 min and the extract filtered. After removal of the EtOH by evapoaration in vacuo at 40°C, the resulting aqueous suspension was centrifuged and then extracted sequentially (each three times) with equal volumes of hexane, diethyl ether (Et_2O), $CHCl_3$, and ethyl-acetate (EtOAc). The post-EtOAc aqueous fraction was concentrated in vacuo at 40°C and adjusted with H_2O to a concentration of 20 gle/ml (1 gle = amount of material extracted from 1 g fresh weight of carrot leaves).

Preparative fractionation of the post-EtOAc aqueous fraction was carried out on an open column (4.1 cm ID) packed with 100.0 g C-18 prep LC packing (Bakerbond, 40-μm particle size). Five milliliters (100 gle) of the post-EtOAc aqueous fraction was applied to the column and eluted successively with 1000 ml 1% acetic acid (HOAc) in H_2O, 1000 ml 20% MeOH in 1% aq. HOAc, 1200 ml 40% MeOH in 1% aq. HOAc, and 500 ml pure MeOH. The brown band that was eluted from the column at the beginning of the pure MeOH fraction was concentrated in vacuo at 40°C and was further fractionated on a second open column (1.9 cm ID × 25.0 cm) packed with C-18 prep LC packing. The column was successively eluted with 275 ml 30% MeOH in 1% aq. HOAc (fraction 1), 460 ml 20% MeOH in 1% aq. HOAc (fraction 2), 230 ml 40% MeOH in 1% aq. HOAc (fraction 3), and 100 ml pure MeOH. The L7MG was collected by repeated HPLC of fraction 3 on a semi-prep C-18 column (Phenomenex IB-sil, 10 × 250 mm, 5 μm). Its identity was confirmed by matching its retention time and UV spectra with the time and spectra of previously isolated luteolin-7-*O*-(6″-*O*-malonyl)-β-D-glucoside (Feeny et al., 1988).

Data Analysis. Statistical comparisons between the regression lines of the surface area correlations for field and greenhouse leaves were carried out using the *F* test for difference between two regression coefficients (Sokal and Rohlf, 1981). Statistical comparisons among the four solvent methods were carried out using the Quade test (Conover, 1980).

RESULTS

Leaf Surface Areas. Although surface area varied linearly with both leaf length and leaf weight, weight was a better predictor of surface area, explaining 98% of the surface area variation in field carrot leaves (Figure 1) and 97% of the variation in greenhouse leaves (Figure 2). The variation of surface area with leaf weight differed significantly between field carrot leaves and greenhouse carrot leaves, as greenhouse leaves had more surface area per gram of leaf ($P < 0.001$, *F* test). The variation of surface area with leaf length also differed significantly between the two groups ($P < 0.01$, *F* test).

Solvent System Comparison. CA, L7G, and L7MG were present in surface extracts obtained using each solvent system, but the systems differed in their

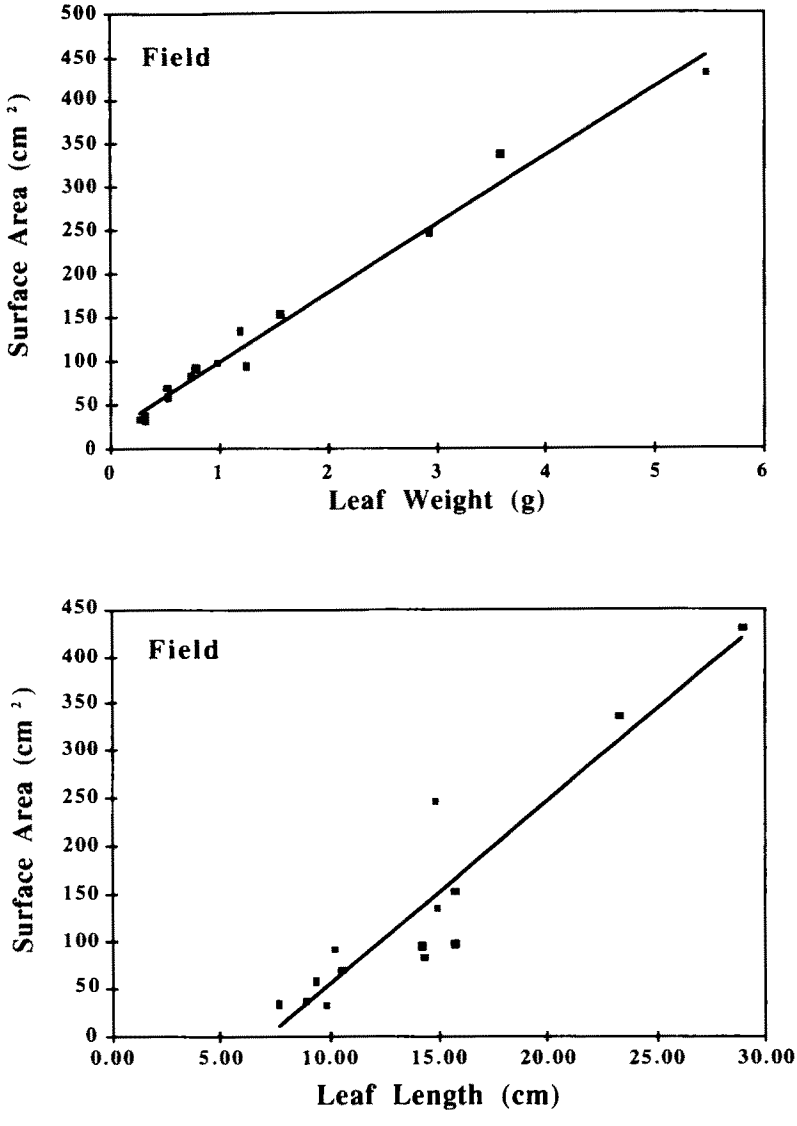

FIG. 1. Relationship of leaf surface area to leaf weight and leaf length, respectively, of field-collected *Daucus carota*. For leaf weight: $y = 78.731\ x + 18.615$ ($r^2 = 0.9827$). For leaf length: $y = 19.231\ x - 139.92$ ($r^2 = 0.8803$).

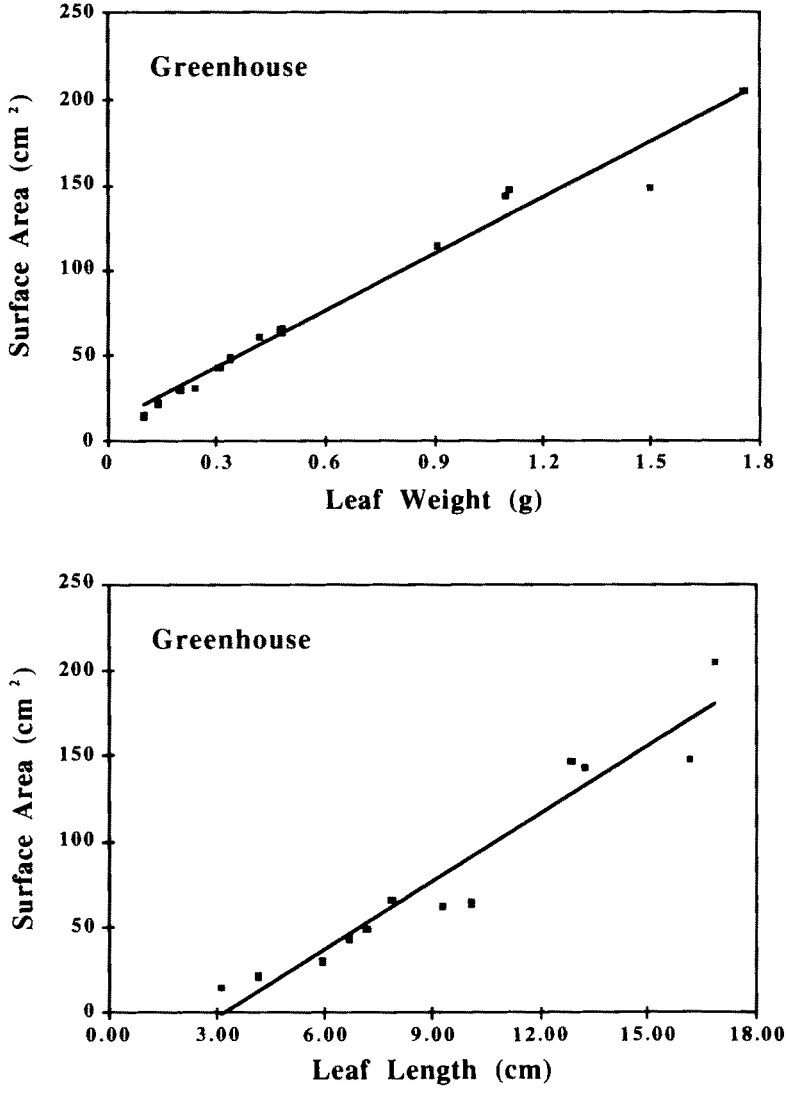

FIG. 2. The relationship of leaf surface area to leaf weight and leaf length, respectively, of greenhouse-grown *Daucus carota*. For leaf weight: $y = 109.37\ x + 9.7964$ ($r^2 = 0.9725$). For leaf length: $y = 13.192\ x - 42.924$ ($r^2 = 0.9263$).

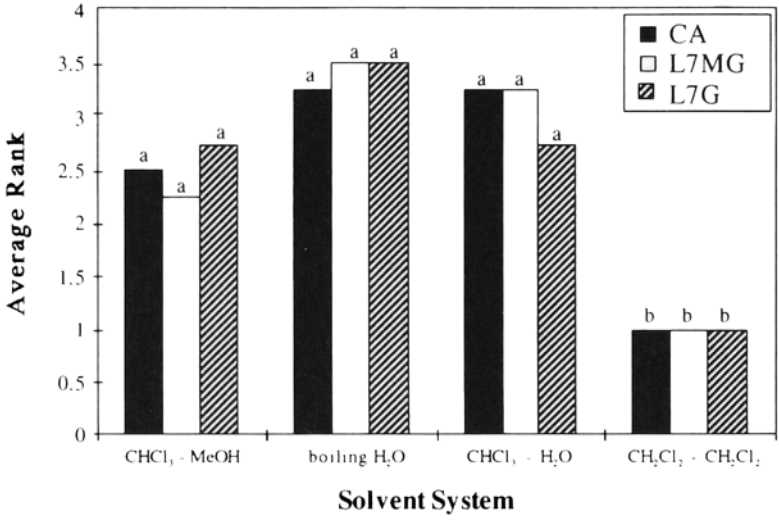

FIG. 3. Comparison of the efficiency of four solvent systems for extracting chlorogenic acid (CA), luteolin-7-*O*-(6″-*O*-malonyl)-β-D-glucoside (L7MG), and luteolin-7-β-D-glucoside (L7G) from leaf surfaces of *Daucus carota*. Efficiency was based on the amounts of a compound detected in surface extracts obtained by each solvent system. The method that removed the highest quantity of a compound received a rank of 4; the method that removed the lowest quantity received a rank of 1. The score given is the average of four replicate samples. Bars with different letters are statistically different ($P < 0.05$, Quade test).

efficiency of extraction (Figure 3). System 4 (CH_2Cl_2–CH_2Cl_2) was significantly less efficient in extracting the polar compounds from the leaf surfaces ($\alpha < 0.05$, Quade test). In fact, no detectable amounts of L7G were found in one of the four methylene chloride samples, and L7MG was not detectable in three of the four methylene chloride samples. System 2 (near-boiling H_2O) and system 3 ($CHCl_3$–near-boiling H_2O) tended to extract higher quantities of the polar compounds from the carrot leaf surfaces than did system 1 ($CHCl_3$–MeOH). The superiority of the aqueous methods was not significant statistically, however, probably due to the low number of replicates in the study.

The four solvent systems also varied in the overall number of polar, UV-absorbing compounds extracted from the carrot leaf surfaces and revealed in HPLC traces. System 3 ($CHCl_3$–near-boiling H_2O) extracted 34 compounds on average from the leaf surfaces. Near-boiling water (system 2) removed 32 compounds on average, and the chloroform–methanol system (system 1) extracted

26 compounds. The methylene chloride solvent system (system 4) extracted an average of only 15 compounds from carrot leaf surfaces.

Compound Quantification. CA was present at much higher concentrations, ranging from 19.2 to 596.2 ng/cm^2, in the $CHCl_3$–near-boiling H_2O surface extracts than were the flavonoid glycosides (Figure 4). This same pattern was also found in the surface extracts in the other three solvent systems. L7G and L7MG were present in extracts at much lower concentrations, ranging from 0.8 to 26.8 ng/cm^2 and 0.9 to 8.2 ng/cm^2, respectively (Figure 4).

There was also seasonal variation in the concentrations of the compounds on the leaf surface (Figure 4). Leaves from the fall rosette foliage had lower concentrations of CA on their surfaces than did the foliage of adult leaves (those from the July and August collections). Although there were also seasonal differences in the concentrations of the flavonoid compounds, no apparent trend was evident from the limited number of sampling dates.

Solvent Timing Study. In the chloroform–near-boiling water solvent system, the amount of time the leaves were dipped in the water affected the amounts of compounds extracted from the leaves (Figure 5), with the quantity extracted from leaves increasing sharply when dipped in water for more than 2 sec.

DISCUSSION

There is debate in the literature as to the possibility of flavonoid glycosides being located on the leaf surface. Harborne (1973) and Wagner (1982) present evidence to show that flavonoid glycosides are confined almost entirely to plant cell vacuoles. However, Hunt and Baker (1980) found naringenin-7-glucoside in the cuticle of tomato fruits and concluded that "flavonoid glycosides occur extensively in the outer epidermis of leaves and fruits but their precise location within this layer has not been clearly established" (p. 1418). Recent studies comparing leaf surface and interior flavonoid composition found flavonoid glycosides in the leaf interior, and only the less polar flavonoid aglycones on the leaf surface (Proksch et al., 1985, 1986; Bohm and Constant, 1990; Bohm and Chan, 1992; Bohm, 1993; Reid and Bohm, 1993). Robinson (1991) described the consensus of research in this area when he stated, "it has been suggested as a general rule that the [flavonoid] glycosides are found within cells" (p. 192).

Although the nonpolar cuticle provides a barrier between the plant leaf interior and the leaf surface, movement of polar compounds through the cuticle has been shown in several plant species. Tukey (1970) documents the diversity of chemicals found in plant leachates, including sugars, sugar alcohols, organic acids, and phenolics. The pesticide literature provides many examples of polar components of applied pesticides moving from the leaf surface into the leaf

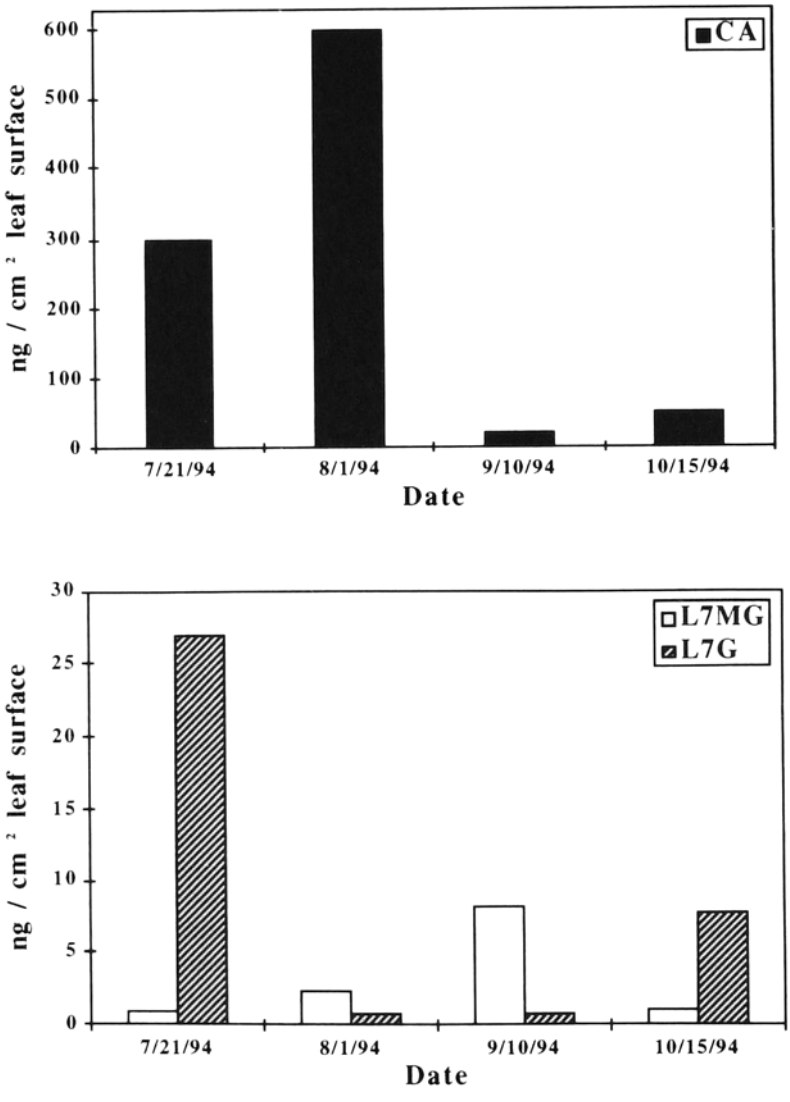

FIG. 4. Quantities of chlorogenic acid (CA), luteolin-7-*O*-(6″-*O*-malonyl)-β-D-glucoside (L7MG), and luteolin-7-β-D-glucoside (L7G) removed from leaf surfaces of *Daucus carota* using the chloroform–near-boiling water method.

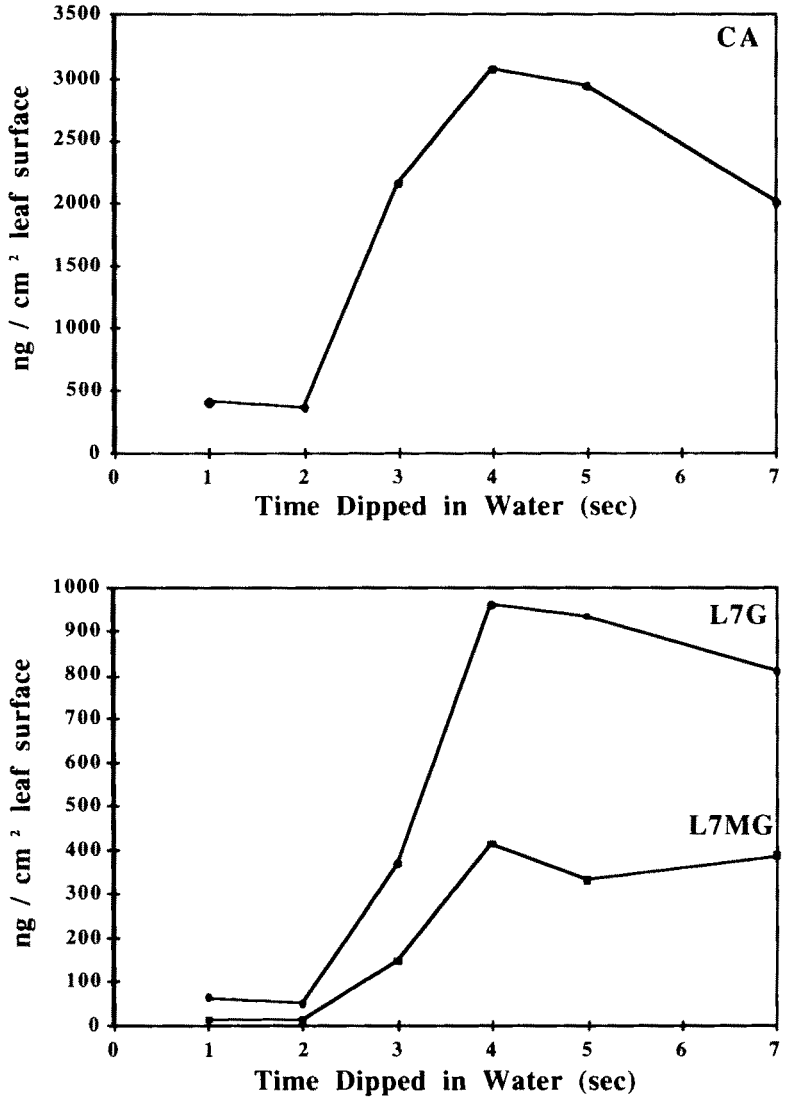

FIG. 5. Quantities of chlorogenic acid (CA), luteolin-7-*O*-(6″-*O*-malonyl)-β-D-glucoside (L7MG), and luteolin-7-β-D-glucoside (L7G) removed from *Daucus carota* leaf surfaces using chloroform and near-boiling water, with successively longer dipping times in water.

interior (see Merrall, 1981; Price, 1982 for references). Merrall (1981) hypothesized that an aqueous or polar pathway existed to explain the facile uptake of hydrophilic and ionic materials that were incompatible with lipids. Price (1982) also reviewed literature support for possible hydrophilic pathways extending from the aqueous apoplast to the cuticle surface but concluded that the evidence in favor of such a theory was rather indirect. More recent work has documented the presence of cuticular pores and transcuticular canals in 63 taxa among 32 families (Miller, 1985, 1986). Derridj et al. (1996) described the kinetics of soluble carbohydrate movement from leaf tissues through the cuticle to the leaf surface. From these studies it can be concluded that the cuticle is not a complete barrier to polar compounds, although the actual mechanism for the passage of polar compounds through the cuticle is unknown.

This current work confirms the presence of the flavonoid glycosides luteolin-7-β-D-glucoside and luteolin-7-*O*-(6″-*O*-malonyl)-β-D-glucoside in leaf surface extracts of *Daucus carota*. The solvent timing study with the chloroform–near-boiling water solvent combination suggests that brief extractions in the water (1–2 sec) remove compounds from the surface alone and not from the apoplast or symplast. It is reasonable to believe that these extracts reflect the presence of the compounds on the leaf surface itself.

Many of the past studies conducted on leaf surfaces used methylene chloride as the solvent for surface extraction (Proksch et al., 1985, 1986; Bohm and Constant, 1990; Bohm and Chan, 1992; Bohm, 1993; Reid and Bohm, 1993). Since methylene chloride significantly underperforms as an extraction solvent in comparison to more polar solvents, and often fails to extract the flavonoid glycosides to any detectable extent, it does not appear to be a suitable solvent for extracting polar compounds from the leaf surface of wild carrot.

Since extraction methods can vary greatly in the amounts of compounds extracted from the leaf surface, it is important to find the method best suited for the compounds of interest to the investigator (see also Städler and Roessingh, 1991; van Loon and van Meer, 1991; Renwick et al., 1992). For extracting the leaf surfaces of *D. carota*, a solvent combination of chloroform and near-boiling water performs somewhat better than other solvents and solvent combinations tested for extraction of polar compounds. It is also a versatile solvent combination that can extract nonpolar compounds in addition to polar compounds. With brief dipping times in water, the method appears to remove compounds from the leaf surface without sampling from the leaf interior to any appreciable extent.

Since swallowtail butterflies make their final oviposition decisions based on leaf surface chemistry, it is important to study chemical stimulants present on the leaf surface itself. Little is yet known concerning the relationship between chemical profiles of leaf surfaces, as perceived by egg-laying females, and the

chemical content of leaf interiors, which are consumed by the feeding larvae. The work described here provides a possible avenue for investigating such relationships.

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DETERMINATION OF FEEDING PREFERENCE OF FORMOSAN SUBTERRANEAN TERMITE (*Coptotermes formosanus* Shiraki) FOR SOME AMINO ACID ADDITIVES

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Abstract—A choice feeding test using 21 amino acids was conducted to determine the feeding preference of Formosan subterranean termite, *Coptotermes formosanus* Shiraki, in the laboratory. Significantly more filter paper treated with D-aspartic acid or L-glutamic acid was consumed by Formosan subterranean termites than was control filter paper treated with water. In two-choice feeding tests, termites consumed significantly more filter paper treated with D-aspartic acid or L-aspartic acid than paper treated with water. Adding L-proline, L-lysine, or L-isoleucine to filter paper significantly increased consumption compared with control filter paper in no-choice tests. The use of amino acid additives in termite baits is briefly discussed.

Key Words—Formosan subterranean termite, *Coptotermes formosanus*, feeding test, amino acids, aspartic acid, proline, glutamic acid.

INTRODUCTION

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is a cosmopolitan pest that attacks both living trees and structural wood (Bess, 1970; Lai et al., 1983). Chemical and physical barriers, wood preservatives, termiticides, and nest excavations are commonly used to control Formosan subterranean termites (Spink, 1967; Su et al., 1991b; Tamashiro et al., 1991; Li et al., 1994). Baiting as a means of controlling subterranean termites was initiated in

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1967 (Esenther and Gray, 1968). Over the years, materials used in the bait matrix have included wood blocks (French and Robinson, 1981), toilet paper rolls (Haverty et al., 1975), corrugated cardboard (La Fage et al., 1983), and cork (French et al., 1986). Since the initiation of termite bait research, considerable time and effort have been spent to find slow-acting and nonrepellent or less repellent bait toxicants (Jones, 1984; Haverty et al., 1989; Su et al., 1991a; Su, 1994), preferred cellulose sources and attractive additives in the bait matrix (Esenther and Beal, 1979), and ways to exploit termite foraging behavior in the bait design (Su, 1994). The aim of all these efforts is to induce termites to preferentially feed on the toxic bait. Thus, the chemistry of the matrix is one of the most important factors in determining bait success.

Generally, termite foods are high in cellulose but low in nitrogen (Brooks, 1963). Because nitrogen is usually limited in naturally occurring termite food (Cowling and Merrill, 1966), nitrogen additives may increase food acceptance and consumption. Hungate (1941) observed that a nitrogen source added to wood with a low nitrogen content increased wood consumption by *Zootermopsis*, *Kalotermes*, and *Reticulitermes* spp. Nitrogen additives also increased the fecundity of mated pairs of *Zootermopsis nevadensis* (Hagen) (Shellman-Reeve, 1990). On a diet of a cellulose (toilet paper) and nitrogen, food consumption by the desert termite, *Gnathamitermes tubiformans* (Buckley), increased as dietary amino acid nitrogen levels increased (Spears and Ueckart, 1976). Mishra (1992) reported that *Neotermes bosei* Snyder (Kalotermitidae) was attracted to proline, aspartic acid, and methionine. Yaga (1972) noted that Formosan subterranean termites consumed filter paper treated with amino acids but failed to include data in his report.

Our goal was to investigate the effect of amino acids on food (filter paper) consumption by Formosan subterranean termites using multiple-choice and no-choice feeding tests. These tests could lead to cheap and effective additives for termite bait enhancement. Feeding preference of termites on D-aspartic acid and L-aspartic acid was also assessed in two-choice feeding tests by using termites from different colonies to determine whether feeding preference remained consistent for termites from different colonies.

METHODS AND MATERIALS

Termites were collected from colonies infesting houses and trees in New Orleans, Lake Charles, and Ascension Parish, Louisiana. Collected termites were kept at room temperature (23–28°C) in plastic containers (20 cm diam., 20 cm height) with sand (No. 4 blasting sand) and moistened corrugated cardboard.

Multiple-Choice Feeding Test. The test apparatus is illustrated in Figure 1.

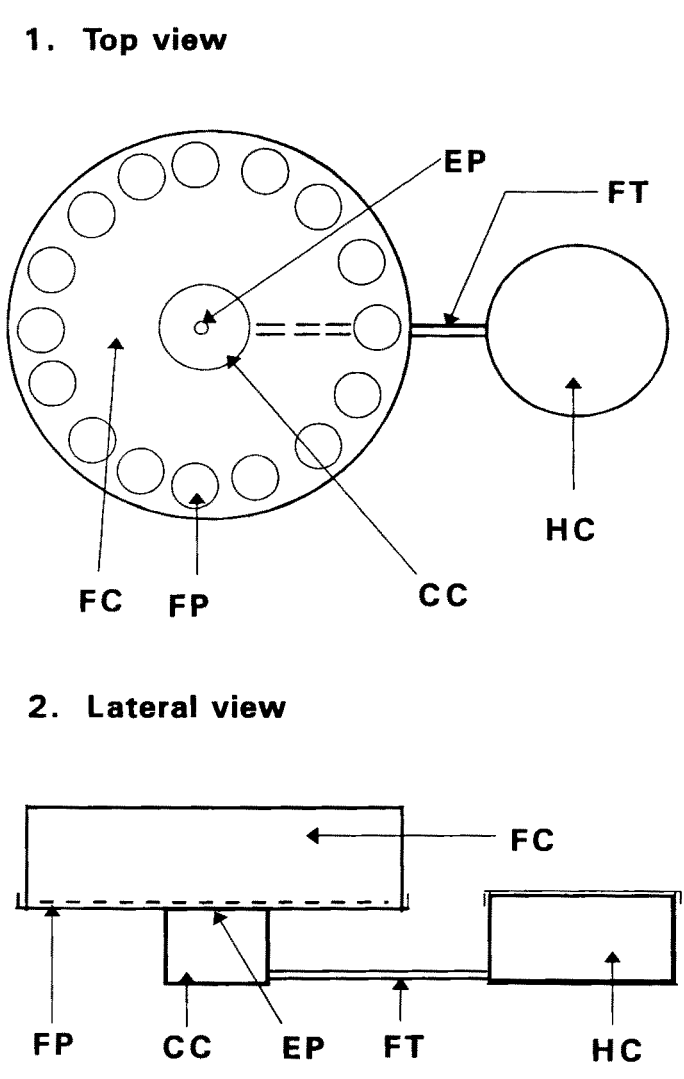

FIG. 1. Choice feeding test apparatus. CC: connecting container, EP: exit path, FC: feeding chamber, FP: filter paper circle, FT: foraging tube, HC: home container.

All termites used in this test came from one colony collected from a home in New Orleans, Louisiana. One hundred fifty workers and 15 soldiers were introduced into a home container (8.41 cm diam., 3.33 cm height, Tristate Plastics, Inc., Dixon, Kentucky) containing 200 g of No. 4 blasting sand that served as

a moisture reservoir. The home container was connected by a foraging tube (0.5 cm inner diam., 13 cm long, Tygon tube) to a connecting container (5.08 cm diam., 3.65 cm height, Tristate Plastics, Inc.) that was filled with moistened sand. A 3-mm-diam. hole (exit path) in the center of the connecting container lid allowed termites to pass upward into a feeding chamber (18.09 cm diam., 7.62 cm depth, Tristate Plastics, Inc.) where the termites had access to food choices consisting of 22 filter paper circles (1.5 cm diam., grade 1, Whatman) fixed to the bottom of the feeding chamber with minute pins. The distance from the exit path to the center of each filter paper circle was 8 cm. After termites were introduced into the home container, the exit path was covered with a piece of Tygon tubing, which prevented the termites from entering the feeding chamber. When termites had tunneled through the home container and connecting container and appeared in the exit path of each apparatus, the tube covering the exit path was removed, so that the feeding tests in all containers ($N = 10$) began simultaneously. Each apparatus was completely buried in moistened vermiculite within a plastic box (31.12 cm long, 22.86 cm wide, and 10.48 cm height, Tristate Plastics, Inc.) to maintain high humidity, decrease outside vibration, and block light.

Amino acids tested were glycine, L-methionine, L-leucine, L-isoleucine, L-cysteine, *trans*-4-hydroxyl-L-proline, *cis*-4-hydroxyl-L-proline, L-lysine, L-proline, L-histidine, L-tryptophan, L-alanine, L-phenylalanine, L-asparagine, L-arginine, L-threonine, L-serine, D-aspartic acid, L-glutamic acid, L-glutamine, and L-valine (purity 99+% by TLC, Sigma Chemical Company, St. Louis, Missouri). Amino acids were dissolved in distilled, deionized water (distilled water supplied through laboratory taps was then deionized using a Barnstead NANOpore Ultrapure Water System) to provide 0.01 M solutions. Solutions that were 0.01 M in strength were selected for testing based on the results of Allsopp's (1992) work on the phagostimulant effect of amino acids on subterranean larvae of *Antitrogus parvulus* and *Lepidiota negatoria* (Coleoptera: Scarabaeidae). Twenty microliters for each amino acid solution were applied to a filter paper circle with a micropipet after it was dried at 80°C for 24 hr, cooled in a desiccator for 5 hr, and weighed. Amino acid solutions and control applications of distilled, deionized water were randomly assigned to a filter paper circle in each feeding chamber ($N = 10$). After five days of termite foraging, filter paper circles were removed. The sand on the filter paper circles, if any, was removed by using a fine forceps. The filter paper circles were dried at 80°C for 24 hr, cooled in a desiccator for 5 hr, and weighed. A completely randomized block design was used with 10 blocks (containers) and 21 amino acids as treatments, and one distilled, deionized water as the control. The response variable was the food consumption (micrograms per termite per day). The 10 replications were conducted simultaneously. The data were analyzed by ANOVA (SAS Institute, 1988), and Tukey's mean comparison was used to compare the dif-

ference in food consumption between the amino acid treatments and control filter paper circles.

Two-Choice Feeding Test. D-Aspartic acid and L-aspartic acid were tested in an experimental apparatus similar to that used in the multiple-choice feeding test, except that the feeding chamber was smaller (8.41 cm diam., 3.33 cm height) and only two food choices were presented to the foraging termites. One filter paper circle was treated with 20 μl of 0.01 M D-aspartic acid or 0.01 M L-aspartic acid (purity 99+% by TLC, Sigma Chemical Company). The other filter paper circle was treated with 20 μl of distilled, deionized water. The four termite colonies tested with D-aspartic acid were collected from four different trees: maple (*Acer* sp.), green ash (*Fraxinus* sp.), elm (*Ulmus* sp.) in New Orleans, Louisiana, and bald cypress [*Taxodium distichum* (L.) Rich.] in Lake Charles, Louisiana. Tests with colonies from maple and green ash were replicated six times, and tests with colonies from elm and bald cypress were replicated 10 times. For L-aspartic acid tests, two termite colonies were collected from houses in Ascension Parish and New Orleans, Louisiana. Tests for both colonies were replicated 10 times. The experimental procedure was the same as that of the multiple-choice feeding test except that the feeding duration was reduced to 48 hr, and 50 worker termites and five soldier termites were used. Because there were only two food choices in the feeding chamber, a paired-sample *t*-test was used to compare the mean consumption of D-aspartic-acid-treated filter paper circles or L-aspartic-acid-treated filter paper circles with filter paper circles treated with distilled, deionized water separately for each colony.

No-Choice Feeding Test. Termites used in this experiment were collected from an infested building in Lake Charles, Louisiana. One hundred worker termites and 10 soldiers were placed in each of 48 plastic Petri dishes (8.41 cm diam., 3.33 cm height, Tristate Plastics, Inc.). Dishes received filter papers (Whatman No. 2, 1.30 $\pm$ 0.007 g dry weight) that were moistened with 2 ml of a 0.01 M amino acid solution of one of 15 amino acids, or distilled, deionized water alone. The experiment was replicated three times (16 treatments $\times$ 3 replications = 48 dishes). Amino acids tested included glycine, L-isoleucine, L-methionine, L-leucine, L-cysteine, *trans*-4-hydroxyl-L-proline, L-lysine, L-proline, L-histidine, L-alanine, L-phenylalanine, L-threonine, L-serine, L-asparagine, and D-aspartic acid (purity 99+% by TLC, Sigma Chemical Company). Termites were held at 27–28°C for 10 days and then filter papers were removed, dried at 80°C for 24 hr, cooled in a desiccator for 5 hr, and weighed. A completely randomized design with three replicates was used with 15 amino acids as treatments and one distilled, deionized water as the control. The response variable was the food consumption (micrograms per termite per day). The data were analyzed by one-way ANOVA. Tukey's mean comparison test was used to compare food consumption among the treated and control filter paper circles.

RESULTS

Multiple-Choice Feeding Test. Mean food consumption (Figure 2) was significantly different between amino acid treatments and control ($F = 2.29$, $df = 21, 189$; $P = 0.002$) but there were no significant differences between the 10 replicates for each treatment ($F = 0.92$, $df = 9, 189$; $P = 0.506$). Of the 21 amino acids tested, only D-aspartic acid or L-glutamic acid significantly increased consumption compared with distilled, deionized water controls ($P < 0.01$, Figure 2). There was no significant difference in food consumption between filter paper circles treated with D-aspartic acid and those treated with L-glutamic acid ($P > 0.05$).

Two-Choice Feeding Test. Termites from each of the four colonies preferentially fed on the filter paper circles treated with D-aspartic acid over filter paper circles treated with distilled, deionized water (Table 1). Termites from each of the other two colonies preferentially fed on the filter paper circles treated with L-aspartic acid over filter paper circles treated with distilled, deionized water (Table 2).

No-Choice Feeding Test. Mean filter paper consumption in the no-choice test was significantly different between amino acid treatments and control ($F =$

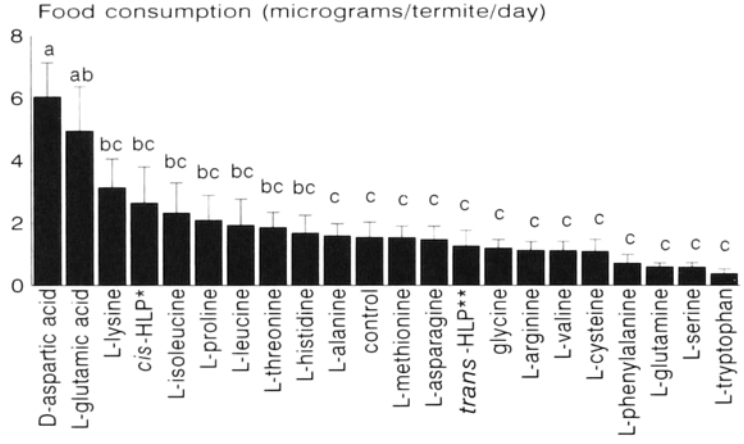

FIG. 2. Mean consumption (+SE) of filter paper circle treated with one of 21 amino acids or distilled, deionized water (control) by Formosan subterranean termites under a multiple-choice bioassay. L-Aspartic acid was not included in this bioassay. Means with the same letter are not significantly different ($P > 0.05$) using Tukey's mean separation. **cis*-HLP: *cis*-4-hydroxyl-L-proline. ***trans*-HLP: *trans*-4-hydroxyl-L-proline.

TABLE 1. FEEDING PREFERENCE BETWEEN FILTER PAPER CIRCLES TREATED WITH D-ASPARTIC ACID (TREATMENT) AND TREATED WITH DISTILLED, DEIONIZED WATER (CONTROL) (MEAN FOOD CONSUMPTION ± SE) BY FORMOSAN SUBTERRANEAN TERMITES COLLECTED FROM LAKE CHARLES AND NEW ORLEANS, LOUISIANA (TEST DURATION: 48 HOURS)

Colony[a]	Loss in dry mass (mg)			Paired-sample *t*-test *P* value
	Treatment	Control	Difference	
1	3.76 ± 0.22	3.13 ± 0.19	+0.63	<0.05
2	3.94 ± 0.40	2.12 ± 0.32	+1.82	<0.01
3	3.32 ± 0.50	1.30 ± 0.18	+2.02	<0.01
4	1.97 ± 0.16	0.70 ± 0.16	+1.27	<0.01

[a]Colony 1 was collected from bald cypress tree in Lake Charles, Louisiana, colonies 2, 3, and 4 were collected from elm, green ash, and maple trees respectively, in New Orleans, Louisiana.

7.35, $df = 15, 32$; $P < 0.01$) (Figure 3). Consumption of filter paper treated with glycine, L-methionine, L-leucine, L-cysteine, *trans*-4-hydroxyl-L-proline, L-histidine, L-alanine, L-phenylalanine, L-threonine, L-serine, L-asparagine, or D-aspartic acid was not significantly different from consumption of filter paper circles treated with distilled, deionized water (Figure 3). Only consumption of filter papers treated with L-proline, L-isoleucine, or L-lysine was significantly greater than consumption of control filter papers ($P < 0.001$ for L-proline, $P = 0.036$ for L-lysine, $P = 0.001$ for L-isoleucine).

TABLE 2. FEEDING PREFERENCE BETWEEN FILTER PAPER CIRCLES TREATED WITH L-ASPARTIC ACID (TREATMENT) AND TREATED WITH DISTILLED, DEIONIZED WATER (CONTROL) (MEAN FOOD CONSUMPTION ± SE) BY FORMOSAN SUBTERRANEAN TERMITES COLLECTED FROM ASCENSION PARISH AND NEW ORLEANS, LOUISIANA (TEST DURATION: 48 HOURS)

Colony[a]	Loss in dry mass (mg)			Paired-sample *t*-test *P* value
	Treatment	Control	Difference	
1	2.51 ± 0.56	0.46 ± 0.32	+2.05	<0.05
2	3.26 ± 0.82	0.55 ± 0.38	+2.71	<0.05

[a]Colony 1 was collected from a house in Ascension Parish, Louisiana, colony 2 was collected from a house in New Orleans, Louisiana.

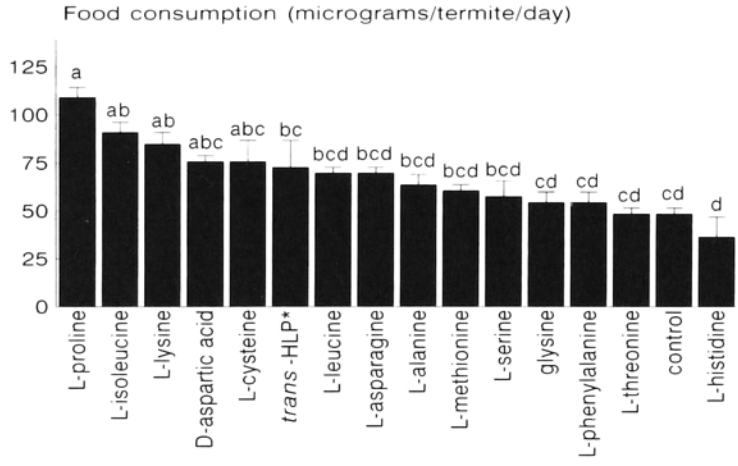

FIG. 3. Mean consumption (+SE) of filter paper circle treated with one of 15 amino acids or distilled, deionized water (control) by Formosan subterranean termites given no choice. L-Aspartic acid was not included in this array. Means with the same letter are not significantly different ($P > 0.05$) using Tukey's mean separation. **trans*-HLP: *trans*-4-hydroxyl-L-proline.

DISCUSSION

Nitrogen is essential in fulfilling life functions of many animals including Formosan subterranean termites. Although there is evidence of nitrogen fixation in termites (Potrikus and Breznak, 1977), whether nitrogen fixation can meet all of the termite's nitrogen requirements remains unanswered. Results of our study indicated that Formosan subterranean termites preferred filter paper containing certain amino acids. This suggests that Formosan subterranean termites may feed preferentially on food sources high in nitrogen and, thus, take a proactive role in providing the colony with needed nitrogen.

Unexpectedly, our experiments also suggest that aspartic acid and glutamic acid may play an important role in the feeding preference of Formosan subterranean termites for sugarcane, which they seriously damage in some areas of south China (Dai and Luo, 1980). Aspartic acid, glutamic acid, and alanine are three of the most common free amino acids in sugarcane raw juice, and aspartic acid and glutamic acid are the most common amino acids in proteins of the cane juice (Irvine, 1985). Sugarcane bagasse (residue of milling or diffusing cane) is used as an additive material to increase the attractiveness of Formosan subterranean termite baits in China (Zhao, 1981). About half (by weight) of the freshly squeezed bagasse is fiber and the remaining weight is residual juice (Chen, 1985).

In nature, D-amino acids are found only in small peptides of bacterial cell walls and in some peptide antibiotics. Although intestinal protozoans and bacteria aid in food digestion by termites, the question of whether termites can utilize D-amino acids as nitrogen sources can be answered only by further experiments. A preliminary study indicates that extended feeding (>30 days) on D-aspartic-acid-treated cardboard is toxic to Formosan subterranean termites (Henderson, unpublished data).

There were differences between the results of our no-choice and choice tests. Inconsistencies between results of choice and no-choice feeding tests have been reported previously (Meehan and Wilde, 1989). Consumption data from no-choice tests must be interpreted carefully since less nutritious food sources sometimes result in increased feeding in order to compensate for the lower nutritional value of a food, as found in tests with lepidopteran larvae fed differing amounts of nitrogen (Slansky and Feeny, 1977; Scriber, 1979). Likewise, data resulting from choice experiments in the laboratory may not always be most likely to represent actual field results (Meehan and Wilde, 1989). We feel that our research indicates that the simple addition of specific amino acids to a termite bait may increase consumption and improve bait efficacy, but this must be tested in the field.

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